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(54) Title: METHODS FOR PRODUCING CAROTENOID COMPOUNDS, AND SPECIALITY OILS IN PLANT SEEDS

(57) Abstract: Methods are provided for producing plants and seeds having altered carotenoid compositions by transforming host plants with constructs having a transcriptional initiation region from a gene expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from at least one carotenoid biosynthesis gene coding region, and a transcriptional termination region. The methods find particular use in increasing the carotenoid content in oilseed plants.

METHODS FOR PRODUCING CAROTENOID COMPOUNDS, AND SPECIALITY OILS IN PLANT SEEDS

This application is a continuation-in-part of Application Serial No. 09/023,587, filed February 13, 1998, and a continuation-in-part of Application Serial No. 09/130,549, filed August 6, 1998, which is a continuation-in-part of Application Serial No.08/908,758 filed August 8, 1997 which claims the benefit of the filing date of provisional Application Serial No.60/024,145 filed August 9, 1996.

FIELD OF THE INVENTION

The invention relates to genetic modification of plants, plant cells and seeds, particularly altering carotenoid biosynthesis, and fatty acid composition.

BACKGROUND OF THE INVENTION

Carotenoids are pigments with a variety of applications. They are yellow-orange-red lipids which are present in green plants, some molds, yeast and bacteria. Carotenoid hydrocarbons are referred to as carotenes, whereas oxygenated derivatives are referred to as xanthophylls. The carotenoids are part of the larger isoprenoid biosynthesis pathway which, in addition to carotenoids, produces such compounds as chlorophyll and tocopherols, Vitamin E active agents. The carotenoid pathway in plants produces carotenes, such as α- and β-carotene, and lycopene, and xanthophylls, such as lutein.

The biosynthesis of carotenoids involves the condensation of two molecules of the C_{20} precursor geranyl PP_i to yield the first C_{40} hydrocarbon phytoene. In a series of sequential desaturations, phytoene yields lycopene. Lycopene is the precursor of the

cyclic carotenes, ß-carotene and α-carotene. The xanthophylls, zeaxanthin and lutein are formed by hydroxylation of ß-carotene and α-carotene, respectively.

ß-carotene, a carotene whose color is in the spectrum ranging from yellow to orange, is present in a large amount in the roots of carrots and in green leaves of plants. ß-carotene is useful as a coloring material and also as a precursor of vitamin A in mammals. Current methods for commercial production of ß-carotene include isolation from carrots, chemical synthesis, and microbial production.

A number of crop plants and a single oilseed crop are known to have substantial levels of carotenoids, and consumption of such natural sources of carotenoids have been indicated as providing various beneficial health effects. The below table provides levels of carotenoids that have been reported for various plant species.

CAROTENOID CONTENTS OF VARIOUS CROPS
(μg/g)

Crop	Beta-Carotene	Alpha-Carotene	Lycopene	Lutein	Total
Carrots	30-110	10-40	0-0.5	0-2	65-120
Pepper (gr)	2	-	-	2	8
Pepper (red)	15	1	-	-	200
Pumpkin	16	0.3	tr	26	100
Tomato	3-6	-	85	-	98
Watemelon	1	tr	19	-	25
Marigold petals	5	4	-	1350	1500
Red palm oil	256	201	8	-	545

The pathway for biosynthesis of the carotenoids has been studied in a variety of organisms and the biosynthetic pathway has been elucidated in organisms ranging from bacteria to higher plants. See, for example, Britton, G. (1988) *Biosynthesis of carotenoids*, p. 133-182, In T.W. Goodwin (ed.), Plant pigments, 1988. Academic

Press, Inc. (London), Ltd., London. Carotenoid biosynthesis genes have also been cloned from a variety of organisms including *Erwinia uredovora* (Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712; *Erwinia herbicola* (Application WO 91/13078, Armstrong *et al.* (1990) *Proc. Natl. Acad. Sci., USA 87*:9975-9979); *R. capsulatus* (Armstrong *et al.* (1989) *Mol. Gen. Genet. 216*:254-268, Romer *et al.* (1993) *Biochem. Biophys. Res. Commun. 196*:1414-1421); *Thermus thermophilus* (Hoshino *et al.* (1993) *Appl. Environ. Microbiol. 59*:3150-3153); the cyanobacterium *Synechococcus sp.* (Genbank accession number X63873). See also, application WO 96/13149 and the references cited therein.

While the genes have been elucidated, little is known about the use of the genes in plants. Investigations have shown that over expression or inhibition of expression of the plant phytoene synthase (Psy1) gene in transgenic plants can alter carotenoid levels in fruits. See, Bird *et al.* (1991) *Biotechnology 9*:635-639; Bramley *et al.* (1992) *Plant J. 2*:343-349; and Fray and Grierson (1993) *Plant Mol. Biol. 22*:589-602. Further, as reported by Fray *et al.* (1995) *The Plant Journal 8*:693-701, constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway.

Application WO 96/13149 reports on enhancing carotenoid accumulation in storage organs such as tubers and roots of genetically engineered plants. The application is directed towards enhancing colored native carotenoid production in specific, predetermined non-photosynthetic storage organs. The examples of the application are drawn to increasing colored carotenoids in transformed carrot roots and in orange flesh potato tubers. Both of these tissues are vegetative tissues, not seeds, and natively have a high level of carotenoids.

Carotenoids are useful in a variety of applications. Generally, carotenoids are useful as supplements, particularly vitamin supplements, as vegetable oil based food products and food ingredients, as feed additives in animal feeds and as colorants.

Specifically, phytoene finds use in treating skin disorders. See, for example, U.S. Patent No. 4,642,318. Lycopene, α- and β-carotene are used as food coloring agents. Consumption of β-carotene and lycopene has also been implicated as having preventative effects against certain kinds of cancers. In addition, lutein consumption has been associated with prevention of macular degeneration of the eye.

Plant oils are useful in a variety of industrial and edible applications. Novel vegetable oils compositions and/or improved means to obtain oils compositions, from biosynthetic or natural plant sources are needed. Depending upon the intended oil use, various different fatty acid compositions are desired. The demand for modified oils with specific fatty acid compositions is great, particularly for oils high in oleic acid. See, Haumann, B. F. (1996) *INFORM* 7:320-334. As reported by Haumann, the ideal frying oil would be a low-saturate, high oleic and low linolenic oil. Furthermore, studies in recent years have established the value of monounsaturated fatty acids as a dietary constituent.

Attempts have been made over the years to improve the fatty acid profiles of particular oils. For example, the oxidative stability of vegetable oil is related to the number of double bonds in its fatty acids. That is, molecules with several double bonds are recognized to be more unstable. Thus, scientists have attempted to reduce the content of α-linolenic acid in order to improve shelf life and oxidative stability, particularly under heat.

It is apparent that there is needed a method for producing significant levels of carotenoid compounds in crop plants and particularly in plant seeds. It would additionally be beneficial to alter the fatty acid content of the plants and seeds. Such altered seed products would be useful nutritionally as well as provide a source for producing more stable oils. There is no report of methods to substantially altering the levels and composition of carotenoids produced in a plant seed, particularly with respect

to increasing the level of production of carotenoids. There is therefore needed, a useful method for altering carotenoid levels in plants, particularly seeds, and for producing oils with modified carotenoid composition and/or content.

SUMMARY OF THE INVENTION

Transformed plants, plant cells and seeds having altered carotenoid levels and/or modified fatty acid compositions are provided. The plants, plant cells and seeds are transformed with at least one carotenoid biosynthesis gene, or a combination thereof. Methods for making and using the transformed compositions of the invention are also provided. Methods find use in altering carotenoid levels in plants, particularly seeds, as well as increasing particular compounds for molecular farming, such as for production of particular carotenoids. At the same time, the transformed compositions, particularly seeds, provide a source of modified oils, which oils may be extracted from the seeds in order to provide an oil product comprising a natural source of various carotenoids, carotenoid mixtures. In a particular aspect of the present invention, transformed seed can provide a source for particular carotenoid compounds and/or for modified specialty oils having altered carotenoid compositions and/or altered fatty acid composition, particularly having increased levels of oleic acid and decreased levels of linoleic and linolenic acids.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence of the SSU/*crt*B fusion sequence, SEQ ID No. 1.

Figure 2 presents constructs for expression of carotenoid biosynthesis genes in plant seeds. Figure 2A shows plasmid pCGN3390 which con

tains the napin promoter operably linked to the SSU/*crtB* sequence. Figure 2B shows plasmid pCGN3392which contains the napin promoter operably linked to the SSU/*crtE* sequence. Figure 2C shows plasmid pCGN9010 which contains the napin promoter operably linked to the SSU/*crtI* sequence. Figure 2D shows plasmid pCGN9009 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to the SSU/*crtI* sequence. Figure 2E shows plasmid pCGN9002 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to an antisense epsilon cyclase sequence. Figure 2F shows plasmid pCGN9017 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to an antisense beta cyclase sequence. Figure 2G shows plasmid pCGN6204 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to the SSU/*crt*W sequence. Figure 2H shows plasmid pCGN6205 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to the *crt*Z sequence. Figure 2I shows plasmid pCGN6206 which contains the napin promoter operably linked to the SSU/*crtB* sequence, the napin promoter operably linked to the *crt*W sequence and the napin promoter operably linked to the *crt*Z sequence. Figure 2J provides a schematic diagram of the corn expression construct pCGN9039.

Figure 3 shows the results of analyses of saponified samples for control seeds.

Figure 4 shows the results of analyses of saponified samples for pCGN3390 transformed seeds.

Figure 5 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates that the increase in 18:1 fatty acids correlates with a decrease in 18:2 and 18:3.

Figure 6 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates that the increase in 18:1 correlates with an increase in both 18:0 and 20:0, but little effect is seen in 16:0.

Figure 7 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates the increase in 18:0 correlates well with an increase in 20:0.

Figure 8 shows a carotenoid biosynthesis pathway.

Figure 9 provides sequence of *B. napus* epsilon cyclase cDNA clone 9-4, SEQ ID No. 2.

Figure10 provides sequence of *B. napus* epsilon cyclase cDNA clone 7-6, SEQ ID No. 3.

Figure 11 provides sequence of a *B. napus* beta cyclase cDNA clone, SEQ ID No. 4.

Figure 12 provides T2 seed analysis of 3390 transformed *Brassica napus* plants.

Figure 13 provides T3 seed analysis of 3390 transformed *Brassica napus* plants.

Figure 14 provides T2 seed analysis of 9002 transformed *Brassica napus* plants.

Figure 15 shows the nucleotide sequence of the SSU/*crt*Z fusion sequence, SEQ ID No. 5, and the deduced amino acid sequence SEQ ID No. 6.

Figure 16 shows the nucleotide sequence of the SSU/*crt*W fusion sequence, SEQ ID No. 7, and the deduced amino acid sequence SEQ ID No. 8.

Figure 17 shows the HPLC trace for detection of xanthophylls from extractions from seed of 6204 transgenic lines.

Figure 18 provides the results of the expression of the maize phytoene synthase in *Arabidopsis* comparing the levels of B-carotene to total carotenoid levels in 9061 lines.

Figure 19 provides the complete nucleic acid sequence of the maize phytoene synthase sequence SEQ ID No. 9, and the deduced amino acid sequence, SEQ ID No. 10.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, methods for increasing production of carotenoid compounds, as well as for altering fatty acid compositions in a plant, particularly in plant seeds, are provided. The method involves transforming a plant cell with at least one carotenoid biosynthesis biosynthesis gene. This has the effect of altering carotenoid biosynthesis, particularly increasing the production of downstream products, as well as providing novel seed oils having desirable fatty acid compositions. A second gene can then be utilized to shunt the metabolic activity to the production of particular carotenoid, or to further alter the fatty acid composition.

Surprisingly, it has been found that transformation of a plant with an early carotenoid biosynthesis gene leads to a significant increase in the flux through the carotenoid pathway resulting in an increase in particular carotenoids. That is, there is an increase in the metabolic activity that can be further manipulated for the production of specific carotenoids. In addition, the transformed seeds may demonstrate altered fatty acid compositions as the result of the carotenoid gene expression, such as seen with the seeds described herein from plants transformed with a phytoene synthase gene.

Thus, using the methods of the invention, seeds are provided which produce high levels of particular carotenoids and/or produce specialty oils having a desired fatty acid composition. In oilseed *Brassica*, for example, transformation with an early carotenoid biosynthesis gene leads to seeds having significant increases in the levels of α-carotene, β-carotene and lutein. In addition, the *Brassica* seeds demonstrate an altered fatty acid composition and yield a vegetable oil which has increased oleic acid content and

decreased linoleic and linolenic acid content. Thus, the transformed seed can provide a source of carotenoid products as well as modified seed oil. In this manner, modified specialty oils can be produced and new sources of carotenoids for extraction and purification are provided.

The oils of the present invention also provide a substantial improvement with respect to stability as compared to two other major plant sources of carotenoids, marigold petals and red palm oil (mesocarp). Although instability is observed in seeds stored in air at room temperature as demonstrated by loss of approximately 20-30% of total carotenoids after 4 weeks of storage, the loss after 1-2 weeks is only 10%. Palm mesocarp, by contrast, must be processed within a day or two of harvest in order to avoid major losses of carotenoids. Furthermore, the carotenoid decomposition in the seeds of the present invention may be reduced significantly by storage of the seeds under nitrogen.

For the production of a seed having an increase in carotenoid biosynthesis, transformation of the plant with an early carotenoid biosynthesis gene is sufficient. By early carotenoid biosynthesis gene is intended geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate (IPP) isomerase. A variety of sources are available for the early carotenoid biosynthesis genes and for the most part, a gene from any source can be utilized. However, it is recognized that because of co-suppression, the use of a plant gene native to the target host plant may not be desirable where increased expression of a particular enzyme is desired.

A number of early carotenoid biosynthesis genes, also referred herein as DNA sequences derived from carotenoid biosynthesis gene coding regions, have been isolated and are available for use in the methods of the present invention. See, for example:

IPP isomerase has been isolated from: *R. Capsulatus* (Hahn *et al.* (1996) *J. Bacteriol. 178*:619-624 and the references cited therein), GenBank Accession Nos.

U48963 and X82627, *Clarkia xantiana* GenBank Accession No. U48962, *Arabidopsis thaliana* GenBank Accession No. U48961, *Schizosaccharmoyces pombe* GenBank Accession No. U21154, human GenBank Accession No. X17025, *Kluyveromyces lactis* GenBank Accession No. X14230;

geranylgeranyl pyrophosphate synthase from *E. Uredovora* Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712 and Application WO 91/13078; and from plant sources, including white lupin (Aitken *et al.* (1995) *Plant Phys.108*:837-838), bell pepper (Badillo *et al.* (1995) *Plant Mol. Biol. 27*:425-428) and *Arabidopsis* (Scolnik and Bartely (1994) *Plant Physiol. 104*:1469-1470; Zhu *et al.* (1997) *Plant Cell Physiol. 38*:357-361).

phytoene synthase from a number of sources including *E. Uredovora, Rhodobacter capsulatus,* and plants Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712, GenBank Accession No. D90087, Application WO 91/13078, Armstrong *et al.* (1989) *Mol. Gen. Genet. 216*:254-268, Armstrong, G. A. "Genetic Analysis and regulation of carotenoid biosynthesis. *In* R. C. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), *Anoxygenic photosynthetic bacteria; advances in photosynthesis.* Kluwer Academic Publishers, Dordrecht, The Netherlands, Armstrong *et al.* (1990) *Proc. Natl. Acad. Sci USA 87*:9975-9979, Armstrong *et al.* (1993) *Methods Enzymol. 214*:297-311, Bartley and Scolnik (1993) *J. Biol. Chem. 268*:27518-27521, Bartley *et al.* (1992) *J. Biol. Chem. 267*:5036-5039, Bramley *et al.* (1992) *Plant J. 2*:291-343, Ray *et al.* (1992) *Plant Mol. Biol. 19*:401-404, Ray *et al.* (1987) *Nucleic Acids Res. 15*:10587, Romer *et al.* (1994) *Biochem. Biophys. Res. Commun. 196*:1414-1421, Karvouni *et al.* (1995) Plant *Molecular Biology 27*:1153-1162, GenBank Accession Nos. U32636, Z37543, L37405, X95596, D58420, U32636, Z37543, X78814, X82458, S71770, L27652, L23424, X68017, L25812, M87280, M38424, X69172, X63873, and X60441, Armstrong, G. A. (1994) *J. Bacteriol. 176*:4795-4802 and the references cited therein; and,

phytoene desaturase from bacterial sources including *E. uredovora* Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712, and Application WO 91/13078 (GenBank Accession Nos. L37405, X95596, D58420, X82458, S71770, and M87280); and from plant sources, including maize (Li *et al.* (1996) *Plant Mol. Biol. 30*:269-279), tomato (Pecker *et al.* (1992) *Proc. Nat. Acad. Sci. 89*:4962-4966 and Aracri *et al.* (1994) *Plant Physiol. 106*:789), and *Capisum annuum* (bell beppers) (Hugueney *et al.* (1992) *J. Biochem. 209*: 399-407), GenBank Accession Nos. U37285, X59948, X78271, and X68058).

See, generally, Misawa *et al.* (1990) *J. of Bacteriology 172*:6704-6712, E.P. 0393690 B1, U.S. Patent No. 5,429,939, Bartley *et al.* (1992) *J. Biol. Chem. 267*:5036-5039, Bird *et al.* (1991) *Biotechnology 9*:635-639, and US Patent No. 5,304,478, which disclosures are herein incorporated by reference.

Transformation with an early carotenoid gene, (herein referred to as the primary gene), increases the biosynthetic activity of the carotenoid pathway, and can lead to increased production of particular carotenoids such as for example, α- and β-carotene. As described in more detail in the following examples, by expression of phytoene synthase as the primary gene, large increases in the carotenoid content generally, and particularly in the levels of α- and β-carotene, are obtained in seeds of transformed plants. Oil comprising the carotenoids so produced may be extracted from the seeds to provide a valuable source of α- and β-carotenes. Such an oil may find use as a food colorant, for example to add color to margarines, or as a food oil. An edible food oil with high α- and β-carotene levels is of interest for prevention of Vitamin A deficiency which can result in night blindness. Thus, production of transformed plants and extraction of the high α- and β-carotene oil to provide a useful food oil is particularly desirable in regions where night blindness is a widespread problem, such as in India and Asia.

In addition to high α- and β-carotene levels, levels of other carotenoids are also increased in the oils exemplified herein. For example, lutein levels are increased in seeds from plants transformed with a phytoene synthase gene, as well as in seeds from plants transformed with a GGPP synthase gene, *crt*E (3392), or with phytoene desaturase, *crt*I (9010).

Furthermore, additional primary genes may be expressed to provide for even greater flux through the carotenoid pathway. For example, in oilseed *Brassica* seeds transformed with a phytoene synthase gene as described herein, increased levels of phytoene are observed. Thus, increasing the expression of phytoene desaturase as well as phytoene synthase may result in further increases in the levels of carotenoids, such as α- and β-carotene and lutein, produced. Such further modification of carotenoid composition is demonstrated here in transgenic plant seeds transformed with pCGN9009 for the expression of *crt*B and *crt*I genes. Additionally, plants expressing both phytoene synthase and GGPP synthase genes are desirable. Such plants may demonstrate even greater flux through the carotenoid pathway as indicated by the increased production of chlorophyll observed in plants of the present invention which have been transformed to express a GGPP synthase gene (*crt*E) in the absence of *crt*B overexpression.

Interestingly, plants expressing a GGPP synthase gene did not have significant modifications of the tocopherol content. Since GGPP is a branch point of the carotenoid, chlorophyll and tocopherol pathways in plants, these observations suggest that the next enzymatic step in tocopherol biosynthesis, catalyzed by GGPP hydrogenase, is a rate limiting step for tocopherol production. Thus, providing for increased expression of GGPP hydrogenase, alone or in conjunction with increased expression of GGPP synthase would be expected to result in an increase of flux to the tocopherol pathway.

Also of interest are plants which are transformed to express three early carotenoid biosynthesis gens, *crt*B, *crt*E, and *crt*I. Plants expressing two or three different carotenoid biosynthsis genes may be produced by either transforming a plant with a construct providing for expression of the desired genes, using a multiple gene construct or by cotransformation with multiple constructs, or by crossing plants which contain the different desired genes.

In addition to the production of the carotenoids described herein, once the biosynthetic activity has been increased by expression of the primary carotenoid biosynthesis gene or genes, the pathway can be diverted for the production of specific compounds. The diversion involves the action of at least one second gene of interest, (the secondary gene). The secondary gene can encode an enzyme to force the production of a particular compound or alternatively can encode a gene to stop the pathway for the accumulation of a particular compound. For forcing the production of a particular compound, expression of a carotenoid biosynthesis gene in the pathway for the desired carotenoid compound is used. Genes native or foreign to the target plant host may find use in such methods, including, for example, carotenoid biosynthesis genes from sources other than higher plant, such as bacteria, including *Erwinia* and *Rhodobacter* species. For stopping the pathway in order to accumulate a particular carotenoid compound, the secondary gene will provide for inhibition of transcription of a gene native to the target host plant, wherein the enzyme encoded by the inhibited gene is capable of modifying the desired carotenoid compound. Inhibition may be achieved by transcription of the native gene to be inhibited in either the sense (cosuppression) or antisense orientation of the gene.

For example, for alteration of the carotenoid composition towards the accumulation of higher levels of ß-carotene derived carotenoids, such as zeaxanthin, zeaxanthin diglucoside, canthaxanthin, and astaxanthin, inhibition of lycopene epsilon

cyclase is desired to prevent accumulation of alpha carotene and its derivative carotenoids, such as lutein. In addition, overexpression of lycopene β-cyclase may be used to increase the accumulation of β-carotene derived carotenoids. Thus, antisense lycopene epsilon cyclase and lycopene β-cyclase are examples of sequences which find use in secondary gene constructs of interest in the present invention. Furthermore, in conjunction with the inhibition of lycopene epsilon cyclase, increased expression of additional secondary genes may be desired for increased accumulation of a particular beta-carotene derived carotenoid. For example, increased β-carotene hydroxylase expression is useful for production of zeaxanthin, wherease increased β-carotene hydroxylase and keto-introducing enzyme expression is useful for production of astaxanthin. Alternatively, for accumulation of lycopene, inhibition of lycopene beta cyclase or of lycopene epsilon cyclase and lycopene beta cyclase is desired to reduce conversion of lycopene to alpha- and beta-carotene.

Thus, the carotenoid pathway can be manipulated by expression of carotenoid biosynthesis genes to increase production of particular carotenoids, or by decreasing levels of a particular carotenoid by transformation with antisense DNA sequences which prevent the conversion of a selected precursor compound into the next carotenoid in the pathway.

Secondary genes of interest in the present application include but are not limited to:

β-carotene hydroxylase or *crt*Z (Hundle *et al.* (1993) *FEBS Lett. 315*:329-334, GenBank Accession No. M87280) for the production of zeaxanthin;

genes encoding keto-introducing enzymes, such as *crt*W (Misawa *et al.* (1995) *J. Bacteriol. 177*:6575-6584, WO 95/18220, WO 96/06172) or ß-C-4-oxygenase (*crt*O; Harker and Hirschberg (1997) *FEBS Lett. 404*:129-134) for the production of canthaxanthin;

crtZ and *crt*W or *crt*O for the production of astaxanthin;

ε-cyclase and ε-hydroxylase for the production of lutein;

ε-hydroxylase and crtZ for the production of lutein and zeaxanthin;

lycopene β-cyclase (crtY) (Hugueney *et al.* (1995) *Plant J.* *8*:417-424, Cunningham FX Jr (1996) *Plant Cell* *8*:1613-1626, Scolnik and Bartley (1995) *Plant Physiol.* *108*:1343, GenBank Accession Nos. X86452, L40176, X81787, U50739 and X74599) for increased production of β-carotene.

antisense lycopene ε-cyclase (GenBank Accession No. U50738) for increased production of β-carotene;

antisense lycopene ε-cyclase and lycopene β-cyclase for the production of lycopene;

antisense plant phytoene desaturase for the production of phytoene; etc.

In this manner, the pathway can be modified for the high production of any particular carotenoid compound of interest, or for a particular subset of carotenoid compounds, such as xanthophylls. Such compounds include but are not limited to the particular compounds described above, as well as, α-cryptoxanthin, β-cryptoxanthin, ζ-carotene, phytofluene, neurosporane, adonixanthin, echineneone, hydroxycanthaxanthin and the like. For a review of xanthophyll production, see Misawa, *et al.* (1995) *supra*). Using the methods of the invention, any compound of interest in the carotenoid pathway can be produced at high levels in a seed.

Secondary genes can also be selected to alter the fatty acid content of the plant for the production of specialty oils. For example, acyl-ACP thioesterase genes having specificity for particular fatty acid chain lengths may be used. See, for example, USPN 5,304,481, USPN 5,455,167, WO 95/13390, WO 94/10288, WO 92/20236, WO 91/16421, WO 97/12047 and WO 96/36719. Other fatty acid biosynthesis genes of interest include, but are not limited to, ß-keto acyl-ACP synthases (USPN 5,510,255),

fatty acyl CoA synthases (USPN 5,455,947), fatty acyl reductases (USPN 5,370,996) and stearoyl-ACP desaturases (WO 91/13972).

Of particular interest is the use of a mangosteen acyl-ACP thioesterase as a secondary gene for fatty acid content modification. As described in WO 96/36719 and WO 97/12047, a high stearate content may be obtained in seeds by expression of a mangosteen acyl-ACP thioesterase. To combine the high oleic acid trait of the 3390 plants described herein with the 5266 high stearate plants described in WO 97/12047, crosses were made between 3390-1 and 5266-35 and between 3390-1 and 5266-5. Seeds resulting from these crosses contained oil having a high stearate, low linoleic, low linolenic and high carotenoid phenotype.

Any means for producing a plant comprising the primary gene or both the primary and secondary genes are encompassed by the present invention. For example, the secondary gene of interest can be used to transform a plant at the same time as the primary gene either by inclusion of both expression constructs in a single transformation vector or by using separate vector, each of which express desired primary or secondary genes. The secondary gene can be introduced into a plant which has already been transformed with the primary gene, or alternatively, transformed plants, one expressing the primary gene and one expressing the secondary gene, can be crossed to bring the genes together in the same plant.

By combining the genes with tissue specific promoters, the carotenoid levels can be altered in particular tissues of the plant. Thus, carotenoid levels in the seed, including embryos and endosperm, can be altered by the use of seed specific transcriptional initiation regions. Such regions are disclosed, for example, in U.S. Patent No. 5,420,034, which disclosure is herein incorporated by reference.

In this manner, the transformed seed provides a factory for the production of modified oils. The modified oil may be used or alternatively, the compounds in the oils

can be isolated. Thus, the present invention allows for the production of particular compounds of interest as well as speciality oils.

The primary or secondary genes encoding the enzymes of interest can be used in expression cassettes for expression in the transformed plant tissues. To alter the carotenoid or fatty acid levels in a plant of interest, the plant is transformed with at least one expression cassette comprising a transcriptional initiation region linked to a gene of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions.

The transcriptional initiation may be native or analogous to the host or foreign or heterologous to the host. By foreign is intended that the transcriptional initiation region is not found the wild-type host into which the transcriptional initiation region is introduced.

Of particular interest are those transcriptional initiation regions associated with storage proteins, such as napin, cruciferin, ß-conglycinin, phaseolin, or the like, and proteins involved in fatty acid biosynthesis, such as acyl carrier protein (ACP). See, U.S. Patent No. 5,420,034, herein incorporated by reference.

The transcriptional cassette will include the in 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau et al., (1991), *Mol. Gen. Genet., 262*:141-144; Proudfoot, (1991), *Cell, 64*:671-674; Sanfacon et al., (1991), *Genes Dev., 5*:141-149; Mogen et al., (1990), *Plant Cell, 2*:1261-1272;

Munroe et al., (1990), *Gene, 91*:151-158; Ballas et al., (1989), *Nucleic Acids Res., 17*:7891-7903; Joshi et al., (1987), *Nucleic Acid Res., 15*:9627-9639).

For the most part, the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression. Thus, the carotenoid biosynthesis gene or genes of interest may be inserted into the plastid for expression with appropriate plastid constructs and regulatory elements. Alternatively, nuclear transformation may be used in which case the expression cassette will contain a gene encoding a transit peptide to direct the carotenoid biosynthesis gene of interest to the plastid. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun. 196*:1414-1421; and, Shah *et al.* (1986) *Science 233*:478-481. Plant carotenoid genes useful in the invention may utilize native or heterologous transit peptides.

It is noted that where the gene or DNA sequence of interest is an antisense DNA, targeting to a plastid is not required. In addition, where antisense inhibition of a given carotenoid biosynthesis gene is desired, the entire DNA sequence derived from the carotenoid biosynthesis gene is not required.

The construct may also include any other necessary regulators such as plant translational consensus sequences (Joshi, C.P., (1987), *Nucleic Acids Research, 15*:6643-6653), introns (Luehrsen and Walbot, (1991), *Mol. Gen. Genet., 225*:81-93) and the like, operably linked to the nucleotide sequence of interest.

It may be beneficial to include 5' leader sequences in the expression cassette which can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T.R., and Moss, B. (1989) *PNAS USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus)

(Allison et al., (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology, 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak, D.G., and Sarnow, P., (1991), *Nature, 353*:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S.A., and Gehrke, L., (1987), *Nature, 325*:622-625; tobacco mosaic virus leader (TMV), (Gallie, D.R. et al., (1989), *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. et al., (1991), *Virology, 81*:382-385. See also, Della-Cioppa et al., (1987), *Plant Physiology, 84*:965-968.

Depending upon where the DNA sequence of interest is to be expressed, it may be desirable to synthesize the sequence with plant preferred codons, or alternatively with chloroplast preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest. See, EPA 0359472; EPA 0385962; WO 91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Research 17*: 477-498. In this manner, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used. For the construction of chloroplast preferred genes, see USPN 5,545,817.

In preparing the transcription cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction,

annealing, resection, ligation, or the like may be employed, where insertions, deletions or substitutions, *e.g.* transitions and transversions, may be involved.

The recombinant DNA molecules of the invention can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) *BioTechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium* mediated transformation (Hinchee *et al.* (1988) *Biotechnology 6*:915-921) and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see, Weissinger *et al.* (1988) *Annual Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37(onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674(soybean); McCabe *et al.* (1988) *Bio/Technology 6*:923-926 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740(rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA, 85*:4305-4309(maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444(maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839; and Gordon-Kamm *et al.* (1990) *Plant Cell 2*:603-618 (maize).

Alternatively, a plant plastid can be transformed directly. Stable transformation of chloroplasts has been reported in higher plants, see, for example, Svab *et al.* (1990) *Proc. Nat'l. Acad. Sci. USA* 87:8526-8530; Svab & Maliga (1993) *Proc. Nat'l Acad. Sci. USA* 90:913-917; Staub & Maliga (1993) *Embo J.* 12:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. In such methods, plastid gene expression can be accomplished by use of a plastid gene promoter or by trans-activation of a silent plastid-borne transgene positioned for expression from a selective

promoter sequence such as that recognized by T7 RNA polymerase. The silent plastid gene is activated by expression of the specific RNA polymerase from a nuclear expression construct and targeting of the polymerase to the plastid by use of a transit peptide. Tissue-specific expression may be obtained in such a method by use of a nuclear-encoded and plastid-directed specific RNA polymerase expressed from a suitable plant tissue specific promoter. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci., USA 91*:7301-7305.

The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.*, *Plant Cell Reports* (1986), 5:81-84. These plants may then be grown, and either self or crossed with a different plant strain, and the resulting homozygotes or hybrids having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

As a host cell, any plant variety may be employed. Of particular interest, are plant species which provide seeds of interest. For the most part, plants will be chosen where the seed is produced in high amounts, a seed-specific product of interest is involved, or the seed or a seed part is edible. Seeds of interest include the oil seeds, such as oilseed *Brassica* seeds, cotton seeds, soybean, safflower, sunflower, coconut, palm, and the like; grain seeds, *e.g.* wheat, barley, oats, amaranth, flax, rye, triticale, rice, corn, etc.; other edible seeds or seeds with edible parts including pumpkin, squash, sesame, poppy, grape, mung beans, peanut, peas, beans, radish, alfalfa, cocoa, coffee, tree nuts such as walnuts, almonds, pecans, chick-peas etc.

It is noted that the methods of the present invention have been demonstrated to provide increased carotenoid production in both oilseed *Brassica*, which has a green embryo, and in cotton, which has a white embryo.

In seed of cotton plants transformed with phytoene synthase, increases of total carotenoid levels ranging from 10 to 300 fold may be obtained. The majority of the increase in carotenoid levels, in this case, about 80%, is observed as an increase in phytoene levels. Increases in lutein levels are also obtained in this case, ranging from 1.5 to a 5 fold increase. In addition, α-carotene and β-carotene levels are also increased 10 to 100 fold, with β-carotene levels being 20 fold higher than α-carotene levels. Thus, as seen with Brassica, a second early carotenoid biosynthesis gene, such as phytoene desaturase, may be used with *crt*B to increase the metabolic flux through the carotenoid/ isoprenoid pathway in cotton to produce a particular carotenoid.

Furthermore, it should also be noted that the methods of the present invention have also been demonstrated herein to provide increased carotenoid production in additional plant species, such as *Arabidopsis* and corn.

In seed of *Arabidopsis* plants transformed with phytoene synthase, increases of total carotenoid levels ranging from 3 to at least approximately 20 fold may be obtained. A large increase in the level of β-carotene, ranging from 10 to 70 fold, are observed in seeds of transgenic *Arabidopsis* plants. Increases in lutein levels are also obtained in this case, ranging from 1.5 to a 3 fold increase. In addition, phytoene, α-carotene and lycopene levels are also increased. However, such increases in α-carotene, phytoene and lycopene are difficult to quantify as these levels are too low to measure in nontransformed control plants. Thus, as seen with Brassica and cotton, a second early carotenoid biosynthesis gene may be used with *crt*B to increase the metabolic flux through the carotenoid/ isoprenoid pathway in cotton to produce a particular carotenoid and to reduce the increased levels of phytoene.

In seed of corn plants transformed with phytoene synthase, increases of total carotenoid levels ranging from 2 to at least approximately 5 fold are obtained. The majority of the increase is seen in the levels of phytoene, while additional increases were

observed in β-carotene levels. Increases of up to about 15 fold increases in β-carotene levels were obtained. Thus, again as with Brassica, cotton, and *Arabidopsis*, a second early carotenoid biosynthesis gene can be employed with the *crt*B to increase the metabolic flux through the carotenoid/ isoprenoid pathway in corn to produce a particular carotenoid and to reduce the increased levels of phytoene. Additionally, additional genes, including secondary carotenoid biosynthesis genes can also be used to direct the production of particular carotenoids and xanthophyls.

In one embodiment of the invention, seed transcriptional initiation regions are used in combination with at least one carotenoid biosynthesis gene. This increases the activity of the carotenoid pathway and alters carotenoid levels in the transformed seed. In this manner, particular genes can be selected to promote the formation of compounds of interest. Where the gene selected is an early carotenoid biosynthesis gene the transformed seed has a significant increase in carotenoid biosynthesis as the result of an increase in the flux through the pathway. For *Brassica* seeds transformed with an early carotenoid biosynthesis gene, significant increases in the production of α-carotene, β-carotene and smaller increases in lutein in the seed oil, as well as altered oil fatty acid compositions are obtained. Seeds obtained from corn plants transformed with an early carotenoid biosynthesis gene also demonstrate an increased amount of carotenoid production.

Where the early carotenoid biosynthesis gene is phytoene synthase, significant increases of a particular carotenoid include those ranging from a 10 to a 50 fold increase, preferably at least a 50 to a 100 fold increase, more preferably, at least a 50 to a 200 fold increase, such as the increases seen in α-carotene and β-carotene levels. Lutein levels, in this case, are also increased, but lower increases of 1.5 - 2 fold are obtained. At the same time, total carotenoid levels may be increased at least 10 to 25 fold, preferably 25 to 60 fold, and more preferably 25 to 100 fold. Thus, a seed of the invention

transformed with a phytoene synthase gene has a substantial increase in levels of α- and β-carotene and total carotenoids, as well as smaller increases in lutein and other carotenoids, including phytoene. In some cases, it is not possible to quantitate the fold increase in a given carotenoid compound, as the levels are too low to detect in seeds from comparable non-transformed plants. In *Brassica napus*, for example, α-cryptoxanthin, lycopene, phytoene and phytofluene are all detected in various levels in seeds transformed with a *crt*B gene, but are not detectable in seeds from untransformed *Brassica napus* plants.

Where the early carotenoid biosynthesis gene is GGPP synthase or phytoene desaturase, 1.5 to 2 fold increases in lutein and ß-carotene have been obtained in at least one transgenic plant for each gene. Lycopene is also detected in seeds from *Brassica napus* plants transformed with a *crt*E (GGPP synthase) gene. Total carotenoids in *crt*E or *crt*I transformants are also increased approximately 2 fold. Chlorophyll levels are also increased in *B. napus* transgenic plants expressing a *crt*E gene suggesting an increase in the levels of geranylgeranyl pyrophosphate (GGPP), which is the branch point substrate for carotenoid, chlorophyll and tocopherol biosynthesis. Increases in chlorophyll levels of 1.5 to 2 fold may be obtained in developing and mature seeds. Thus, also of interest as sources of carotenoids are plants which have been engineered to express increased levels of both *crt*B and *crt*E.

As demonstrated herein, the effect of one early carotenoid biosynthesis gene on the metabolic energy flux through the carotenoid pathway may be further effected by the addition of a second early carotenoid biosynthesis gene. Thus, the addition of a second early carotenoid biosynthesis gene for increasing the metabolic flow through the carotenoid biosynthesis pathway is also of interest in the present invention, and may find use for production of particular carotenoids either in the presence or absence of a secondary carotenoid biosynthesis gene.

Where the early carotenoid biosynthesis gene phytoene synthase is cotransformed into *Brassica napus* with a second early carotenoid biosynthesis gene, phytoene desaturase, significant increases of particular carotenoids include increases in α-carotene, β-carotene, and lutein such as observed by expression of *crt*B alone. In addition, lycopene and phytoene levels are also increased in such plants, but increases are difficult to quantitate as these levels are too low to be detected in untransformed *Brassica napus* plants.

Furthermore, when *crt*I and *crt*B are both expressed, total carotenoid levels greater than those observed with *crt*B alone may be obtained. In at least one plant, total carotenoid levels of 1.5 fold those observed in *crt*B plants were obtained. Lycopene levels are also increased over levels obtained in seeds of plants transformed with *crt*B alone. Lycopene levels may be increased from 4 to 15 fold over those obtained in seed of a homozygous *crt*B plant. In addition, a reduction in the ratio of phytoene to total carotenoids is also obtained, and as a result, levels of α-carotene and β-carotene are increased 1.2 to 1.8 fold over those obtained with *crt*B alone. In seeds of plants transformed with phytoene synthase alone, phytoene levels constituted as much as 20% of total carotenoids, while in plants cotransformed with phytoene synthase and phytoene desaturase, phytoene levels represent only 4% to 7% of the total carotenoids.

This metabolic energy effected by transformation with an early carotenoid gene can be funneled into a metabolic compound of choice by transformation with a second gene. As discussed above, the second gene is designed to promote the synthesis of a particular carotenoid by promoting the formation of the carotenoid of interest or alternatively by stopping the pathway to allow for the buildup of compounds. Therefore, significant amounts of carotenoids of interest can be produced in the transformed seeds of the present invention.

Where the primary carotenoid biosynthesis gene phytoene synthase is cotransformed with a secondary carotenoid biosynthesis gene, β-carotene ketolase, increases in levels of α-carotene, β-carotene and phytoene, such as those seen with transformation with *crt*B alone, are obtained. Furthermore, echinenone and canthaxanthin levels are also increased. However, such increases are difficult to quantitate as echinenone and canthaxanthin are either not produced in *Brassica napus*, or the levels are too low to be detected in *B. napus* plants expressing phytoene synthase alone and nontransformed control plants. Thus, for the production of a specific carotenoid, such as astaxanthin, the addition of a third carotenoid biosynthesis gene, such as β-carotene hydroxylase (*crt*Z), may find use in the present invention. Furthermore, the addition of a fourth carotenoid biosynthesis gene, such as phytoene desaturase, may also find use in the present invention.

It should be noted that the carotenoid echinenone is a reaction intermediate in the production of canthoxanthin from β-carotene. The β-carotene ketolase (*crt*W) could react with the β-ring of α- or β-carotene. One β-ring reaction in β-carotene results in echinenone, two β-ring reactions in β-carotene form canthaxanthin, and one β-ring reaction in α-carotene makes 4-keto-α-carotene. This enzyme can not react with the ε-ring of α-carotene. Thus, two additional peaks on the HPLC chromatogram are produced in similar amounts, one representing echinenenone, and the other may represent 4-keto-α-carotene.

Where the primary carotenoid biosynthesis gene phytoene synthase is cotransformed with an antisense secondary carotenoid biosynthsis gene, ε-cyclase, large increases in levels of α-carotene, β-carotene and phytoene, such as those seen with transformation with *crt*B alone, are obtained. Some difference in the ratio of β-carotene to α-carotene is observed as compared to plants transformed with *crt*B alone, but large increases in both α-carotene and β-carotene levels are still observed. Lutein levels, on

the other hand, are either unchanged, increased, or in some cases decreased by as much as 80% as compared to seeds of untransformed control plants.

Initiation of carotenoid biosynthesis begins at approximately 15 days post anthesis in *B. napus* seeds, while expression of transformed genes utilizing the napin promoter begins about 18 days post anthesis. Thus, in order to more tightly control the α-carotene pathway to allow for the build up of β-carotene pathway carotenoids using antisense ε-cyclase, an earlier promoter, such as that of the *Lesquerella* kappa hyrodoxylase (described in pending U.S. patent application 08/898,038, filed 18 July, 1997), may find use. Thus, for increasing levels of a particular carotenoid using antisense, an earlier seed specific tanscriptional initiation region, may be used with a secondary carotenoid biosynthesis gene.

The seeds of the invention which have been transformed with the primary early carotenoid biosynthesis gene also provide a source for novel oil compositions. The use of phytoene synthase as the primary gene, for example, results in substantial increases in oleic acid content in seed oil. By substantial increase is intended an increase of from about 5% to about 40%, specifically from about 20% to about 40%, more specifically from about 30% to about 40%. Thus, the seeds of the invention which have been transformed with a primary early carotenoid biosynthesis gene provide a source for modified oils having a high oleic acid content. That is, carotenoid biosynthesis genes, particularly early carotenoid biosynthesis genes can be used to produce seeds having at least 70% oleic acid, on a weight percentage basis. The oleic acid content in any seed can be altered by the present methods, even those seeds having a naturally high oleic acid content. Alteration of seeds having naturally high oleic acid contents by the present methods can result in total oleic acid contents of as high as 80%.

Importantly, there is also a decrease in linoleic and linolenic acid content. By decrease in linoleic fatty acid content is intended a decrease from about 10% to about

25%, preferably about 25% to about 40%, more preferably about 35% to about 60%. By decrease in linolenic fatty acid content is intended a decrease from about 10% to about 30%, preferably about 30% to about 60%, more preferably about 50% to about 75%. Thus, the methods of the invention result in oils which are more oxidatively stable than the naturally occurring oils. The modified oils of the invention are low-saturate, high oleic and low linolenic. Furthermore, the present invention provides oils high in monounsaturated fatty acids which are important as a dietary constituent.

Based on the methods disclosed herein, seed oil can be modified to engineer an oil with a high oleic acid content as well as a high level of a carotenoid of interest. High oleic acid and high α- and β-carotene oils would have a longer shelf life as both the oleic acid and α- and β-carotene content would lend stability. It is also noted that such oils are more desirable as sources of carotenoids than the natural red palm oil, which oil contains high levels of saturated fatty acids.

The transformed seed of the invention can thus provide a source of carotenoid products as well as modified fatty acids. Where the intent is to produce particular carotenoid compounds of interest, methods are available in the art for the purification of the carotenoid compounds. In the same manner, methods available in the art can be utilized to produce oils purified of carotenoids. See, generally, WO 96/13149 and Favati *et al.* (1988) *J. Food Sci. 53*:1532 and the references cited therein.

The transformed seed and embryos additionally find use as screenable markers. That is, transformed seed and embryos can be visually determined and selected based on color as a result of the increased carotenoid content. The transformed seeds or embryos display a color ranging from yellow to orange to red as a result of the increased carotenoid levels. Therefore, where plant transformation methods involve an embryonic stage, such as in transformation of cotton or soybean, the carotenoid gene can be used in plant transformation experiments as a marker gene to allow for visual selection of

transformants. Likewise, segregating seed can easily be identified as described further in the examples.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1 Expression Construct and Plant Transformation

A. SSU fusions to *E. uredovora* carotenoid biosynthesis genes

(1) Phytoene Synthase

The SSU leader and *crt*B gene sequences were joined by PCR. The sequence of the SSU/*crt*B fusion is shown in Figure 1. The *crt*B gene from nucleotides 5057 to 5363 (numbering according to Misawa *et al.* (1990) *supra*) was joined to the SSU leader as follows. A *Bgl*II site was included upstream of the SSU leader start site to facilitate cloning. The thymidine nucleotide at 5057 of *crt*B was changed to an adenosine to make the first amino acid at the SSU leader/*crtB* junction a methionine, and the splice site a cys-met-asn. The native splice site for SSU is csy-met-gln. Note that Misawa *et al.* (1990) *supra*) indicates that the start site for the coding region for *crtB* is at nucleotide 5096. Thus, there are 13 amino acids upstream of the published start of the coding region for *crtB* and after the SSU splice site in the *crt*B/SSU fusion. Twelve of these amino acids are translated from *Erwinia crt*B upstream sequence and one is the added methionine. The *crtB* from 5363 (*Eco*RV) to 6009 (*Eco*RI) was then attached to the SSU-*crtB* fusion to obtain a complete SSU-*crtB* fusion construct designated pCGN3373 (Fig. 1).

(2) Phytoene Desaturase

A plasmid comprising a *E. uredovora crt*I gene fused to the transit peptide sequence of the pea Rubisco small subunit was described by Misawa *et al.* (*The Plant Journal* (1993) *4*:833-840. An approximately 2.1 kb *Xba*I/*Eco*RI fragment of this plasmid containing the SSU-*crtI* fusion and a nos 3' termination region was cloned in position for expression from a napin 5' promoter.

(3) GGPP Synthase

A similar construct containing the SSU transit fused to an *E. uredovora crt*E gene was obtained. The SSU-*crt*E fusion is present on an approximately 1.2 kb *Bgl*II/*Bam*HI fragment in pCGN3360.

B. SSU fusions to *A. auriantiacum* carotenoid biosynthesis genes

(4) beta-Carotene Hydroxylase (crtZ)

The SSU leader and *crtZ* gene sequences were joined by PCR . The crtZ gene (Misawa, *et al.* (1995) *supra*) nucleotide sequence was resynthesized to adjust for plant codon usage. The re synthesized crtZ gene was joined to the SSU leader by PCR as follows. A BglII site was included upstream of the SSU leader translation start site and a XhoI site was included downstream of the crtZ stop codon to facilitate cloning in the napin expression cassette. The nucleotide sequence of the complete ssu:crtZ fusion is shown in Figure 15.

(5) beta-Carotene Ketolase (crtW)

The SSU leader and *crt*W gene sequences were joined by PCR . The crtW gene (Misawa, *et al.* (1995) *supra*) nucleotide sequence was resynthesized to adjust for plant codon usage. The re synthesized crtW gene was joined to the SSU leader by PCR as follows. A BglII site was included upstream of the SSU leader translation start site and a XhoI site was included downstream of the stop codon to facilitate cloning in the napin expression cassette. The nucleotide sequence of the complete ssu:crtW fusion is shown in Figure 16.

C. Expression Constructs for Plant Transformation

(1) Phytoene Synthase

pCGN3373 carrying the complete SSU/*crtB* fusion was cut with *Bgl*II and *Bam*HI to excise the SSU/*crtB* fusion. The resulting fragment was ligated into the napin expression cassette in pCGN3223 at the BamHI site (see WO 94/10288 for description of napin expression cassette). The resulting construct, pCGN3389, was digested with

*Hin*dIII to excise the napin 5'-SSU/*crtB*-napin 3' fragment, which was then cloned into *Hin*dIII cut pCGN1559PASS yielding pCGN3390. pCGN1559PASS is a binary vector for *Agrobacterium*-mediated transformation such as those described by McBride *et al.* (*Plant Mol. Biol.* (1990) *14*:269-276) and is prepared from pCGN1559 by substitution of the pCGN1559 linker region with a linker region containing the following restriction digestion sites: *Asp*718/*Asc*I/*Pac*I/*Xba*I/ *Bam*HI/*Swa*I/*Sse*8387(*Pst*I)/*Hin*dIII. A map of pCGN3390 is provided in Figure 2A. For expression of phytoene synthase in the corn endosperm, the *crt*B coding sequence from *E. herbicola* (Application WO 91/13078, Armstrong *et al.* (1990) *supra*) was cloned to be expressed under control from the rice glutelin, pGt1, promoter (Leisy, D.J. et al., Plant Mol. Biol. 14 (1989) 41-50) and the HSP70 intron sequence (U.S. Patent Number 5,593,874). This cassette also includes the transcriptional termination region downstream of the cloning site of nopaline synthase, *nos* 3' (Depicker et al., *J. Molec. Appl. Genet.* (1982) 1: 562-573) to create the vector pCGN9039 (Figure 2J) for transformation into corn.

The phytoene synthase coding sequence from corn (Figure 19) was also PCR amplified, fused with the SSU leader and cloned to be expressed from the napin promoter to create the expression construct pCGN9061.

(2) Phytoene Desaturase

A fragment comprising a napin 5'/SSU-*crtI* fusion/nos 3' construct as described above was cloned into a binary vector for plant transformation resulting in pCGN9010. A map of pCGN9010 is provided in Figure 2C.

(3) GGPP Synthase

pCGN3360 carrying the complete SSU/*crtE* fusion was cut with *Bgl*II and *Bam*HI to excise the SSU/*crtE* fusion. The resulting 1.2 kb fragment was ligated into the napin expression cassette in pCGN3223 at the BamHI site. The resulting construct,

pCGN3391, was digested with *Hin*dIII to excise the napin promoter-SSU/*crtE* napin 3' fragment, which was then cloned into *Hin*dIII cut pCGN1559PASS yielding pCGN3392. A map of pCGN3392 is provided in Figure 2B.

(4) Phytoene Synthase + Phytoene Desaturase

The napin 5'-SSU/*crtB*-napin 3' fragment from pCGN3389 and the napin 5'/SSU-*crtI* fusion/nos 3' as present in pCGN9010 were inserted into a binary vector resulting in pCGN9009, shown in Figure 2D.

(5) Antisense Epsilon Cyclase + Phytoene Synthase

Brassica napus epsilon cyclase genes are isolated by PCR using primers designed from an *Arabidopsis* epsilon cyclase gene (Cunningham FX Jr (1996) *Plant Cell 8*:1613-1626). Sequence of *B. napus* epsilon cyclase genes is provided in Figures 9 (clone 9-4) and 10 (clone 7-6). An antisense construct is prepared by cloning an*Xho*I/*Bam*HI fragment of cDNA clone 9-4 into a napin expression cassette (pCGN3223) digested with *Xho*I and *Bgl*II. The napin 5'-antisense epsilon cyclase-napin 3' fragment is cloned along with a napin 5'-SSU/*crtB*-napin 3' fragment, fragment into a binary vector for plant transformation, resulting in pCGN9002, shown in Figure 2E.

(6) Antisense Beta Cyclase + Phytoene Synthase

Brassica napus beta cyclase genes are isolated by PCR using primers designed from an *Arabidopsis* beta cyclase gene (Cunningham FX Jr (1996) *Plant Cell 8*:1613-1626). Sequence of a *B. napus* beta cyclase cDNA, 32-3, is provided in Figures 11. An antisense construct is prepared by cloning an*Xho*I fragment of the beta cyclase cDNA clone into a napin expression cassette (pCGN3223) digested with *Xho*I. A clone containing the beta cyclase in the antisense orientation is selected. The napin 5'-antisense beta cyclase-napin 3' fragment is cloned along with a napin 5'-SSU/*crtB*-napin

3' fragment into a binary vector for plant transformation, resulting in pCGN9017, shown in Figure 2F.

(7) beta-Carotene Hydroxylase + Phytoene synthase

The vector pCGN9003 was constructed by removing the restriction sites between the *crt*B coding sequence and the napin 3' sequence by digestion with *Cla*I and *Xho*I and filling the ends with klenow creating the vector pCGN9000. PCGN9000 was digested with *Asp*718, and the fragment containing the napin 5'/SSU:*crt*B/napin 3' was ligated into the binary vector pCGN5139.

A binary vector for plant transformation, pCGN5139, was constructed using the neomycin phospho-transferase (nptII) kanamycin resistance gene driven by the CAMV 35S transcriptional initiation region (35S 5') and transcription termination (35S 3') sequences (Fraley et al., *Proc. Natl. Acad. Sci* (1983) 80:4803-4807, Gardner *et al.*, (1986) *Plant Mol Biol* 6:221-228). The 35S 5'-nptII-35S 3' fragment was then cloned into a vector containing ori322, Right border (0.5Kb), lacZ, Left Border (0.58Kb), as an *Xho* I fragment between the Right border-lacZ and Left border sequences. The ColEI and pRi origins of replication as well as the Gentamycin resistance gene were aquired from a derivative of pCGN1532 (McBride and Summerfelt, *Plant Molecular Biology*, (1990), 14:269-276). Finally, a linker containing unique restriction sites was synthesized and cloned into the *Asp* 718/ *Hind* III (within the lacZ sequence) sites to create the binary vector pCGN5139.

The plastid targeted ssu:crtZ fusion was cloned into the napin pCGN3223 seed expression cassette as a Bgl II -Xho I fragment to generate pCGN6203. The plasmid pCGN6203 carrying the complete napin cassette with ssu:crtZ was digested with NotI to excise the napin cassette containing the ssu:crtZ coding region. The excised fragment was ligated into the Not I site of the binary pCGN9003 carrying the napin SSU:crtB construct. The resulting construct, pCGN6205 (Figure 2H)

is a binary vector for Agrobacterium-mediated transformation such as those described by McBride et al. (Plant Mol. Biol. (1990) 14:269-276) and is prepared from pCGN1559 by substitution of the pCGN1559 linker region with a linker region containing the following restriction digestion sites: Asp718/AscI/PacI/XbaI/ BamHI/SwaI/Sse8387(PstI)/HindIII. A map of pCGN6205 is provided in Figure 2H.

(8) beta-Carotene Ketolase + Phytoene synthase

The ssu crtW plastid targeted fusion was cloned into the napin pCGN3223 seed expression cassette as a Bgl II -Xho I fragment to generate plasmid pCGN6202.

The plasmid pCGN6202 carrying the napin cassette with ssu:crtW was digested with NotI to excise a DNA fragment containing the napin cassette with ssu:crtZ. The resulting fragment was ligated into the Not I site of the binary pCGN9003 (described above) carrying the SSU:crtB napin construct . The resulting pCGN6204 (Figure 2G) is a binary vector for Agrobacterium-mediated transformation such as those described by McBride et al. (Plant Mol. Biol. (1990) 14:269-276) and is prepared from pCGN1559 by substitution of the pCGN1559 linker region with a linker region containing the following restriction digestion sites: Asp718/AscI/PacI/XbaI/ BamHI/SwaI/Sse8387(PstI)/HindIII. A map of pCGN6204 is provided in Figure 2G.

(9) Phytoene synthase+ beta-Carotene hydroxylase+ beta-Carotene Ketolase

Construct pCGN6203 containing the napin cassette and ssu:crtZ was digested with Hind III to excise the fragment containing napin ssu:crtZ. The resulting HindIII fragment was cloned into the Hind III site of pCGN6204 to generate a triple crt genes binary pCGN6206 that contains napin ssu:crtB+ napin ssu:crtW+ napinssu:crtZ (Figure 2I).

D. Plant Transformation

Transformed *Brassica napus* plants containing the above described constructs are obtained as described in Radke *et al.* (*Theor. Appl. Genet.* (1988) *75*:685-694 and *Plant Cell Reports* (1992) *11*:499-505).

Transformed cotton plants, *Gossypium hirsutum,* containing phytoene synthase may be obtained using methods described in issued U.S. patent No. 5,004,863, and 5,159,135, and in Umbeck *et al.* (1987) *Bio/Technology* 5:263-266, or as described in copending application 08/539,176.

Transgenic *Arabidopsis thaliana* plants containing phytoene synthase may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) *85*:5536-5540), or as described by Bent et al. ((1994), *Science* 265:1856-1860), or Bechtold et al. ((1993), *C.R.Acad.Sci, Life Sciences* 316:1194-1199).

Microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology 10*:286-291) may also be used to obtain nuclear transformed plants.

Example 2 Analysis of Transgenic Plants

A. Visual Observations and Segregation Ratios

The napin-SSU leader/*crtB* plants in 212/86 were tagged at 21 days, 28 days and 35 days post anthesis. When the first plant, 3390-1 was harvested at 28 days, some of the seeds were obviously orange. AT 35dpa, the orange was obvious enough that a segregation ratio could be obtained. This trend of orange seeds has continued and is seen in each of the 17 lines harvested that have been obtained. A table of the segregation ratios is included below in Table 3.

TABLE 3

Generation	Plant #	Orange	Green	Ratio	Chi Square
T2	3390-1	291	88	3 to 1	0.64
T2	3390-2	150	22	No fit	
T2	3390-8	293	87	3 to 1	0.90
T2	3390-4	277	82	3 to 1	0.89
T2	3390-5	243	62	3 to 1	1.90
T2	3390-7	236	89	3 to 1	0.99
T2	3390-6	307	5	63 to 1	0.00
T2	3390-3	121	50	No fit	1.64
T2	3390-11	294	105	3 to 1	0.37
T2	3390-15	287	83	3 to 1	1.30
T2	3390-16	187	65	3 to 1	0.08
T2	3390-17	105	104	No fit	
T2	3390-12	119	28	3 to 1	2.78
T2	3390-14	283	107	3 to 1	1.23
T2	3390-19	238	94	3 to 1	1.94
T2	3390-20	251	4	63 to 1	0.00
T2	3390-27	229	4	63 to 1	0.04

B. Carotenoid Analysis of Developing Seeds

Carotenoids were extracted from seeds harvested at approximately 35 days post-anthesis as follows. Eight seed samples of orange seeds from transgenic plant 3390-1 and eight seed samples of a 212/86 variety rapeseed control plant were ground in 200µl of 70% acetone/30% methanol. The ground seed mixture was then spun in a microcentrifuge for approximately 5 minutes and the supernatant removed. Two additional 70% acetone/30% methanol extractions were conducted with the pelleted seed material and all three supernatants pooled and labeled A/M extract.

At this point in the extraction, the control seed pellets are white, whereas the seed pellets from the transgenic seeds have a yellow color. The pellets are then extracted twice with ether and the resultant supernatants pooled and labeled E extract. The A/M extract was then transferred to ether as follows. 450µl ether and 600µl of water were added to the extracts, followed by removal of the ether layers. The A/M extracts were then washed two more time with 400µl of ether, and the ether fractions from the three A/M washes pooled. The E extracts described above were washed once with 400µl of water and pooled with the A/M ether fractions. The pooled ether fractions were blown down to a volume of approximately 300µl with nitrogen gas and filtered using a syringe microfilter. The sample vials were rinsed with approximately 100µl ether and the rinse was similarly filtered and pooled with the initial filtrate, yielding total volume of approximately 150µl. A 50µl aliquot was stored at -20YC until further analysis and the remaining 100µl sample was saponified as follows. 100µl of 10% potassium hydroxide (KOH) in methanol was added to each 100µl sample and the mixture stored in the dark at room temperature for approximately 2 hours. 400µl of water was then added to the samples and the ether phase removed. For better phase separation, saturated NaCl may be substituted for the water. The water solution was then extracted twice more with 100µl of ether and the ether samples pooled and washed with water.

The saponified samples were then analyzed by HPLC analysis on a Rainin microsorb C18 column (25cm length, 4.6mm outside diameter) at a flow rate of 1.5ml per minute. The gradient used for elution is as follows:

A = acetonitrile

B = hexane/methylene chloride (1:1)

C = methanol.

The initial solution was 70:20:10 (A:B:C). At 2.5 minutes the solution is ramped over 5 minutes to 65:25:10 (A:B:C) and held at this for 12.5 minutes. The solution is then ramped to 70:20:10 (A:B:C) over two minutes followed by a three minute delay prior to injection of the next sample. The absorbance of the eluting samples is continuously monitored at 450 and 280 nm and known chemical and biological standards were used to identify the various absorbance peaks.

In Figures 3 and 4, results of analyses of saponified samples are provided for control and pCGN3390 transformed seeds, respectively. Clear increases in the levels of α- and ß-carotene and phytoene in the transagenic plant seeds are observed, as well as smaller increases in levels of the hydroxylated carotenoid, lutein.

C. Carotenoid and Tocopherol Analysis of Mature Seeds from *crt*B Transgenic Plants

Mature 3390 T2 seed were sent to an analytical laboratory for quantitative analysis using standard HPLC methods known in the art. These results of these analysis are shown in Table 4 below. Compound levels are presented as μg/g.

Seeds designated "Maroon" were selected based on seed color. The seeds which have orange embryos appear maroon colored at maturity as opposed to the black-brown appearance of seeds from wild type plants of this cultivar. Seeds designated as "Random" were not selected for color. As 3390-1 is segregating 3 to 1 for Kan, the "Random" population includes a proportion of nulls. The maroon population contains only transgenics. Due to an effort to exclude nulls from this population, the inclusion of homozygotes may be favored.

TABLE 4

COMPOUND	CONTROL	3390-1 RANDOM	3390-1 MAROON
Lutein	7.2	18	26
Zeaxanthin	nd*	nd	nd
α-cryptoxanthin	nd	8	15
β-cryptoxanthin	nd	nd	nd
Lycopene	nd	2.3	5.1
cis-Lycopene	nd	2.9	5.4
α-carotene	0.6	124	244
β-carotene	0.9	177	338
cis-ß-carotene	0.2	12	26
Other	6	34	51
Total colored carotenoids	14.9	378.2	710.5
Phytoene	nd	62	139
Phytofluene	nd	24	54
Total all carotenoids	14.9	464.2	903.5
Alpha-tocopherol	74	93	109
Gamma-tocopherol	246	188	95
Delta-tocopherol	3	5	5

*nd = not detected

In the non-transgenic sample, "other" includes mostly very polar compounds, such as neoxanthin, violaxanthin, etc. In the transgenic sample "other" includes these and additional compounds, such as zeta-carotene, neurosporene, and mono-cyclic carotenoids.

Results of carotenoid analysis of 3390 T2 seeds from transformed plants of *B. napus* variety Quantum (SP30021) are presented in Figure 12.

Results of carotenoid analysis of 3390 T3 seeds from transformed plants of *B. napus* variety 212/86 (SP001) are presented in Figure 13.

The above results demonstrate that α- and β-carotenes levels are significantly increased in the mature seeds as the result of expression of the *crt*B gene. Generally, the overall increase in carotenoids is quite high, nearly 50 fold for colored carotenoids and up to 60 fold if phytoene and phytofluene are included. It is clear that the flux through the isoprenoid pathway has been dramatically increased. Additionally it is noted that the α-tocopherol (Vitamin E) levels are also increased by nearly 50%.

D. Germination Studies

Ten mature seeds of 3390-1 and 10 seeds of 212/86 control were planted in soil and grown in a walk-in growth chamber. The transgenics emerged 1 to 2 days later than the controls, however, all 10 seeds did germinate. The transgenics were yellowish-pink when they first emerged but greened up in one to two days. At the emergence of the first true leaf, no difference in color was observed. Plants germinated from both the transgenic and control seeds developed normally.

E. Fatty Acid Analysis

Fatty acid composition of mature seeds was determined by GC analysis of single T2 seeds harvested from trangenic plants 3390-1 and 3390-8. Single seeds from both Random (R) and Maroon (M) populations (as defined above) were analyzed and compared to seeds from a 212/86 control (SP001-1). The results of these analyses are provided in Table 5 below as weight % total fatty acids.

TABLE 5

FATTY ACID COMPOSITION OF 3390-1 AND 3390-8 LINES

SAMPLE	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
CONTROL	1.5	0	0.1	5.1	0.4	1.7	59.9	17.1	12.0	0.6	1.2	0.1	0.3
CONTROL	1.8	0	0.1	5.1	0.4	1.7	60.1	16.6	12.1	0.6	1.2	0.1	0.3
CONTROL	2.0	0	0.1	5.0	0.4	1.6	60.5	16.2	12.0	0.6	1.2	0.1	0.3
CONTROL	2.2	0	0.1	5.2	0.4	1.6	57.2	18.2	12.7	0.6	1.3	0.1	0.4
CONTROL	1.6	0	0.1	4.7	0.4	1.8	62.7	15.3	11.3	0.6	1.2	0.1	0.3
3390-1-R	2.8	0	0.1	4.8	0.5	3.6	69.9	10.6	4.8	1.2	1.1	0.0	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.5	58.1	19.3	12.3	0.5	1.2	0.1	0.3
3390-1-R	3.5	0	0.1	4.2	0.3	2.6	71.1	9.6	5.8	1.0	1.2	0.0	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.9	61.0	17.8	10.4	0.7	1.3	0.1	0.3
3390-1-R	2.2	0	0.1	4.4	0.3	3.1	73.6	8.9	4.4	1.2	1.1	0.0	0.7
3390-1-R	1.9	0	0.1	4.5	0.3	2.4	72.7	10.6	4.7	0.9	1.3	0.1	0.6
3390-1-R	2.5	0	0.1	4.2	0.3	3.4	71.7	10.0	5.1	1.1	1.0	0.0	0.6
3390-1-R	1.7	0	0.1	4.4	0.3	2.6	73.5	10.0	4.5	1.0	1.2	0.1	0.6
3390-1-R	1.9	0	0.1	4.2	0.3	2.3	72.4	9.9	6.3	0.9	1.2	0.1	0.5
3390-1-R	2.5	0	0.1	4.2	0.3	2.7	72.0	10.1	5.1	1.0	1.2	0.1	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.7	58.5	18.5	12.6	0.6	1.2	0.1	0.3
3390-1-R	2.8	0	0.1	4.6	0.4	3.7	71.8	9.1	4.2	1.3	1.2	0.0	0.7
3390-1-R	1.8	0	0.1	4.0	0.3	2.3	72.4	11.1	5.2	0.9	1.3	0.1	0.5
3390-1-R	1.7	0	0.1	4.4	0.3	2.7	73.9	9.9	4.2	1.0	1.2	0.1	0.6
3390-1-R	1.7	0	0.1	4.6	0.4	2.6	71.4	10.9	5.5	1.0	1.3	0.1	0.6
3390-1-R	2.7	0	0.1	4.2	0.3	2.8	72.1	9.9	5.0	1.1	1.3	0.0	0.6
3390-1-R	2.0	0	0.1	4.5	0.3	3.0	72.5	9.7	4.6	1.2	1.3	0.1	0.7
3390-1-R	1.8	0	0.1	4.9	0.4	3.4	71.8	10.4	4.2	1.2	1.2	0.0	0.7
3390-1-R*	0.9	0	0.1	4.5	0.3	1.7	55.9	18.8	15.6	0.5	1.3	0.1	0.3
3390-1-R*	1.4	0	0.1	4.8	0.4	1.7	57.1	18.0	14.4	0.6	1.2	0.1	0.3
3390-1-R*	1.4	0	0.1	4.5	0.3	1.7	57.8	18.5	13.5	0.6	1.3	0.1	0.3
3390-1-R	2.2	0	0.1	4.5	0.3	2.5	73.4	9.7	4.6	0.9	1.2	0.0	0.5
3390-1-R	1.5	0	0.1	3.8	0.3	2.7	75.9	8.1	4.6	1.0	1.4	0.0	0.6
3390-1-R	1.6	0	0.1	4.5	0.3	2.6	71.9	10.6	5.5	1.0	1.3	0.1	0.6
3390-1-R*	1.3	0	0.1	6.2	0.5	1.4	53.6	21.7	13.2	0.5	1.1	0.1	0.3
3390-1-R	2.1	0	0.1	4.3	0.3	2.4	72.3	10.7	5.1	0.9	1.2	0.0	0.6
3390-1-R*	1.3	0	0.1	5.0	0.3	1.6	57.8	18.8	13.0	0.5	1.3	0.1	0.3
3390-1-R	2.1	0	0.1	4.4	0.3	3.3	72.7	9.2	4.8	1.2	1.2	0.0	0.7
3390-1-R	1.5	0	0.1	4.5	0.3	3.3	72.6	10.1	4.6	1.2	1.1	0.1	0.7
3390-1-R*	1.2	0	0.1	4.7	0.3	1.9	59.9	17.1	12.6	0.6	1.3	0.1	0.4
3390-1-M	2.8	0	0.1	4.0	0.3	2.8	69.8	10.6	7.1	0.9	1.2	0.0	0.4
3390-1-M	2.0	0	0.1	4.9	0.4	3.3	70.3	11.1	4.9	1.2	1.2	0.1	0.7
3390-1-M	1.5	0	0.1	4.4	0.3	3.2	73.4	9.5	4.3	1.3	1.3	0.0	0.8
3390-1-M	1.5	0	0.1	4.5	0.3	2.8	72.7	10.0	5.1	1.1	1.3	0.0	0.7
3390-1-M	1.8	0	0.1	4.2	0.3	3.1	73.5	9.6	4.7	1.1	1.2	0.0	0.6
3390-1-M	1.5	0	0.1	4.7	0.3	2.9	71.6	10.7	5.1	1.1	1.2	0.1	0.7
3390-1-M	1.5	0	0.1	4.5	0.3	3.2	72.6	10.2	4.3	1.2	1.3	0.0	0.8
3390-1-M	1.8	0	0.1	4.4	0.3	2.9	72.0	10.4	5.2	1.1	1.2	0.1	0.6
3390-1-M	1.5	0	0.1	4.4	0.3	2.6	73.6	10.0	4.5	1.1	1.2	0.1	0.7
3390-1-M	2.3	0	0.1	4.3	0.3	3.0	73.0	9.7	4.5	1.1	1.2	0.0	0.6

SAMPLE	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
3390-8-R*	1.0	0	0.1	4.9	0.3	1.6	59.2	18.9	11.9	0.5	1.2	0.1	0.3
3390-8-R	2.1	0	0.1	4.2	0.3	2.7	71.9	10.2	5.6	1.0	1.2	0.1	0.6
3390-8-R	1.5	0	0.1	4.4	0.3	2.3	72.5	10.4	5.7	0.9	1.4	0.1	0.6
3390-8-R*	1.2	0	0.1	4.9	0.3	1.7	59.7	18.2	11.6	0.6	1.3	0.1	0.4
3390-8-R*	1.5	0	0.1	4.7	0.3	1.6	58.7	18.5	12.2	0.6	1.3	0.1	0.4
3390-8-R	1.8	0	0.1	4.2	0.3	2.9	73.4	9.2	5.2	1.1	1.3	0.0	0.6
3390-8-R*	1.1	0	0.1	4.7	0.3	1.5	56.9	19.3	14.1	0.5	1.1	0.1	0.2
3390-8-R	2.2	0	0.1	4.6	0.3	3.0	71.4	10.0	5.2	1.1	1.2	0.1	0.7
3390-8-R	1.7	0	0.1	4.6	0.4	2.4	72.5	11.0	4.8	0.9	1.3	0.1	0.5
3390-8-R	2.4	0	0.1	4.7	0.3	2.9	74.0	8.4	4.0	1.1	1.2	0.0	0.7
3390-8-R	1.9	0	0.1	4.6	0.4	3.0	72.7	9.7	4.8	1.0	1.2	0.0	0.6
3390-8-R	2.0	0	0.1	4.4	0.3	2.8	73.2	9.7	4.5	1.0	1.3	0.0	0.6
3390-8-R	1.5	0	0.1	4.3	0.3	2.6	71.8	10.7	5.8	1.0	1.3	0.1	0.6
3390-8-R	1.5	0	0.1	4.4	0.3	2.7	72.6	10.5	4.9	1.0	1.3	0.1	0.6
3390-8-R	2.0	0	0.1	4.9	0.4	3.3	71.1	10.4	4.9	1.1	1.1	0.1	0.6
3390-8-R	2.1	0	0.0	4.5	0.4	3.6	73.0	8.8	4.3	1.3	1.2	0.0	0.7
3390-8-R	2.2	0	0.1	5.1	0.4	2.9	67.6	12.3	6.5	1.1	1.2	0.1	0.7
3390-8-R	1.8	0	0.1	4.2	0.3	2.6	73.5	9.9	4.8	1.0	1.3	0.1	0.6
3390-8-R	1.7	0	0.1	4.7	0.3	3.0	72.5	9.9	4.6	1.2	1.3	0.1	0.7
3390-8-R	1.7	0	0.1	4.6	0.4	2.8	73.7	9.5	4.1	1.1	1.3	0.1	0.7
3390-8-R	1.5	0	0.1	4.5	0.3	3.0	74.7	8.5	4.2	1.2	1.2	0.0	0.7
3390-8-R	1.5	0	0.1	4.4	0.4	1.9	70.0	11.8	7.2	0.8	1.4	0.1	0.5
3390-8-R	1.7	0	0.1	4.4	0.3	2.5	71.8	11.1	5.2	1.0	1.3	0.1	0.6
3390-8-R	1.4	0	0.1	4.5	0.4	2.8	73.3	9.7	4.9	1.1	1.2	0.1	0.6
3390-8-R	1.5	0	0.1	4.8	0.4	3.0	72.6	10.6	4.1	1.1	1.2	0.1	0.7
3390-8-R*	1.4	0	0.1	5.8	0.4	2.9	54.0	20.0	13.0	0.8	1.1	0.1	0.4
3390-8-R	1.4	0	0.1	4.4	0.3	2.7	71.2	10.8	6.0	1.0	1.3	0.1	0.6
3390-8-R	1.7	0	0.1	4.6	0.4	2.8	72.6	10.0	5.1	1.0	1.2	0.1	0.6
3390-8-R*	1.0	0	0.1	4.6	0.3	1.6	59.6	18.5	12.3	0.5	1.2	0.1	0.3
3390-8-R*	1.1	0	0.1	4.6	0.3	1.4	56.5	20.4	13.4	0.5	1.3	0.1	0.3
3390-8-M	1.8	0	0.1	4.7	0.4	3.3	70.1	11.1	5.5	1.2	1.1	0.1	0.7
3390-8-M	1.5	0	0.1	4.3	0.3	3.0	73.0	10.3	4.3	1.1	1.2	0.1	0.7
3390-8-M	1.9	0	0.1	4.5	0.4	3.7	73.1	8.9	4.2	1.3	1.2	0.0	0.7
3390-8-M	1.6	0	0.1	4.4	0.3	2.5	73.4	9.7	5.1	1.0	1.3	0.1	0.7
3390-8-M	1.3	0	0.1	4.4	0.3	3.0	73.7	9.6	4.4	1.1	1.3	0.0	0.7
3390-8-M	2.1	0	0.1	4.3	0.3	3.2	74.0	8.9	4.1	1.2	1.2	0.1	0.6
3390-8-M	2.1	0	0.1	3.9	0.3	1.6	71.6	11.9	5.7	0.7	1.5	0.1	0.5
3390-8-M	1.6	0	0.1	4.6	0.3	2.8	71.0	11.8	4.8	1.0	1.3	0.1	0.6
3390-8-M	2.1	0	0.1	4.8	0.4	3.2	70.3	10.7	5.2	1.2	1.2	0.1	0.7
3390-8-M	1.6	0	0.1	4.5	0.3	2.9	72.7	9.9	4.8	1.1	1.3	0.0	0.7

The above data demonstrate a substantial increase in oleic acid (18:1) in seeds from each of the transgenic lines. The increase in oleic acid is at the expense of linoleic and linolenic acids, both of which were decreased in the transgenic lines. Increases in 18:0 and 20:0 fatty acids were also observed. Based on these data, the null seeds present in the Random population can be identified, and are marked on Table 5 with an asterisk (*). All of the seeds in the Maroon populations from each transgenic line have the observed altered fatty acid composition, confirming that the altered fatty acid composition is the result of expression of the *crt*B gene.

The trends in fatty acid composition data in the transgenic seeds which indicate positive and negative correlations of fatty acid composition changes with the observed increase in 18:1 levels are provided in Figures 5-7. The increase in 18:1 correlates with the decreases in 18:2 and 18:3. (Figure 5). The increase in 18:1 also correlates with an increase in both 18:0 and 20:0, but little effect on 16:0 was seen (Figure 6). The increase in 18:0 also correlated with an increase in 20:0 (Figure 7).

F. Carotenoid Analysis of Mature Seeds from *crt*E Transgenic Plants

Carotenoids were analyzed in mature T2 seeds of 3392 *B. napus* plants tranformed to express the *E. uredovora crt*E gene. Approximately two fold increases in levels of lutein and ß-carotene was observed in seeds of plant 3392-SP30021-16. Lycopene was also detected in these seeds and is undetectable in seeds of untransformed control plants. Analysis of seeds from 7 additional 3392 transformants did not reveal significant increases in the carotenoid levels.

G. Analysis of Chlorophyll and Tocopherol Levels in *crt*E Transgenic Plants

Chlorophyll levels were analyzed using a spectrophotometric assay (Bruinsma, J. 1961, A comment on the spectrophotometric determination of chlororphyll, Biochem Biophy Acta, 52:576-578) in mature T2 seeds of transgenic 3392 *B. napus* plants. Levels in 3392 transgenic plants were compared to seeds of transgenic *B. napus* plants

expressing phytoene synthase (crtB) and to nontransformed control plants. Results are shown in Table 6 below.

TABLE 6

Gene and sample	Pigment concentration (μg/gFW) Total carotenoids	Total chlorophyll
Phytoene synthase		
27 DPA SP001 control	53	676
27 DPA T4 3390-1-6	354	282
40 DPA SP001 control	47	471
40 DPA T4 3390-1-6	534	179
50 DPA SP001 control	16	125
50 DPA T4 3390-1-6	648	125
GGPP synthase		
35 DPA SP30021 control	68	407
35 DPA T2 3392-4	65	660
35 DPA T2 3392-16	73	648
Mature SP30021 control	21	35
Mature T2 3392-4	25	31
Mature T2 3392-16	50	60

Chlorophyll concentrations of the 35 DPA seeds of two lines were increased by approximately 60% compared to the levels of the control plant. The initial results demonstrate that the GGPP synthase gene increased the GGPP substrate availability for chlorophyll biosynthesis during seed development. Mature seeds of the 3392-16 line had higher chlorophyll and carotenoid concentrations than those of the control.

H. Carotenoid Analysis of Mature Seeds from *crt*I Transgenic Plants.

Carotenoids were analyzed in mature T2 seeds of 9010 *B. napus* plants tranformed to express the antisense lycopene ε-cyclase gene. Seeds of nine transgenic plants were analyzed for carotenoid content. An approximately two fold increase in levels of lutein, ß-carotene and total carotenoids was observed in seeds of one line, 9010-SP30021-10, when compared to control plants.

I. Carotenoid Analysis of Mature Seeds from *crtB* + *crtI* Transgenic Plants

Carotenoid levels of Mature 9009 T2 seeds were extracted and quantified on an HPLC as follows. Approximately 100mg of seeds were ground in a mortar and pestle in 3ml extraction solvent (hexane/acetone/ethanol (50/25/25 v/v) with 0.2ml of an internal standard (5mg/ml β-apo-8` carotenal (dissolved in 100µl hexane), in acetonitrile/methylene chloride/methanol (50/40/10, v/v/)). The extraction solution was transferred to a new glass tube, and the remaining seed was again extracted with the extraction solvent and pooled with first extraction solution. The extraction was repeated until no color was visible in the extraction solution. Pooled extracts were mixed by vortexing briefly, then centrifuged for approximately 5 minutes. The resulting supernatant was transfered to a new tube and dried under nitrogen gas. The residue was resuspended in 2ml of hexane. Potassium hydroxide, in methanol, was added to a final concentration of 5%, and the solution was incubated overnight in the dark at 4°C. Another 2ml of hexane was then added to the solution with 1ml of saturated sodium chloride. The solution was mixed briefly by vortexing and centrifuged for approximately 5 minutes. The upper hexane layer was removed and transferred to a new glass tube. The remaining bottom phase was again extracted with hexane and centrifuged. The upper phase was combined with the previous hexane phase. This was repeated until the hexane phase was colorless. The pooled hexane phases were dried under nitrogen gas, and the residue was dissolved in 2.0ml of acetonitrile/methylene chloride/methanol (50/40/10 v/v). The solution was filtered through a 0.45µm filter and colected in a brown autopsampler vial. Carotenoid concentrations were determined on a Hewlett Packard 1050 High-Performance Liquid Chromatograph (HPLC), and isocratic separation of carotenoids was performed on a Hewlett Packard reverse phase C-18 (5µ) column (4.6 mm x 20cm) at 30°C. The mobile phase was acetonitrile/ methylene chloride/ methanol (80/10/10, v/v) with a flow rate of 1.0ml/min and a sample injection

volume of 20µl (running time of 22min). Routine detection of colored carotenoids is at 450 nm, phytoene at 280 nm, and phytofluene at 365 nm. Spectral scans for peak purity were made at 250 nm and 600 nm. Spectra of peaks at the upslope, apex, and downslope are normalized and overlaid. Superimposing spectra were taken as evidence of peak purity. The results are shown in Table 7 below. Carotenoid levels are presented as µg/gFW.

TABLE 7

Sample ID #	Lutein	Lycopene	α-Carotene	β-Carotene	Phytoene	Total
SP30021 control	36	ND	ND	4	ND	40
3390-SP001-1-6-15 (T5 Homo)	54	4	552	638	277	1525
9009-SP30021-1	44	44	336	691	42	1157
9009-SP30021-6	53	87	689	1118	152	2099
9009-SP30021-9	48	34	487	798	194	1561
9009-SP30021-10	33	25	248	489	34	829
9009-SP30021-12	31	ND	ND	2	ND	33
9009-SP30021-14	42	37	404	791	81	1355
9009-SP30021-15	37	15	137	278	ND	467
9009-SP30021-16	50	38	428	828	65	1409

The results demonstrate that as with plants transformed to express *crt*B alone, plants expressing *crt*B and *crt*I contain significant increases in total carotenoid levels. Furthermore, it is apparent that expression of *crt*I with *crt*B, leads to further modification of the phytoene pools which accumulate in *crt*B transformants. Phytoene levels were reduced from about 20% of total carotenoids in lines transformed with *crt*B alone, to 4% to 7% of total carotenoids in the crtB + crtI lines. This indicates that phytoene desaturase can have a synergistic effect with phytoene synthase in increasing the metabolic flux through the carotenoid/ isoprenoid pathway, and provides for even

greater increases in a desired carotenoid compound, such as α-carotene and β-carotene, than is obtained by expression of *crt*B alone. The increased flux also appears to result in increased total carotenoid production, in addition to the composition shift from phytoene. For example, the carotenoid levels in the segregating T2 seed populations of 9009-10 are significantly higher than those detected in the 3390 homozygous seed population in 3390-1-6-15.

J. Carotenoid Analysis of mature Seeds from *crt*B + Antisense ε-Cyclase Transgenic Plants

Carotenoids from mature seeds from 9002 transformants were extracted and analyzed using the method described in example 2I above. These results are shown in Figure 14.

The initial results show a modification to the ratio of β-carotene to α-carotene. In addition, several lines show a significant reduction in lutein levels when compared to nontransgenic controls. In 9002 T2 lines, β-carotene to α-carotene ratios averaged 1.5, ranging from 1.1 to 2.5. For comparison, T2 3390 lines containing *crt*B, the ratio of β-carotene to α-carotene averaged 1.9, ranging from 1.5 to 2.4.

K. Carotenoid Analysis of Mature Seeds from *crtB* Transgenic Cotton Plants

Mature 3390 T2 seeds from cotton were collected and carotenoid extracts were prepared and analyzed according to the method described in 2I above. These results are shown in Table 8 below. Carotenoid levels are presented as μg/gFW.

TABLE 8

Sample ID #	Lutein	Lycopene	α-Carotene	β-Carotene	Phytoene	Total
C130 control	2	ND	ND	ND	ND	2
3390-C130-5-1	7	ND	486	420	517	

An approximately 3 fold increase in lutein was observed in seeds of plant 3390-C130-5-1. Alpha-carotene, β-carotene and phytoene were also observed in this line and are undetectable in nontransformed control plants. With β-carotenoid levels being 20 fold higher than those of α-carotene. Total carotenoid levels were increased by more than 250 fold, with phytoene accounting for approximately 80% of that total.

L. Carotenoid Analysis of Mature Seed from *crt*B + *crt*W Transgenic Plants

Carotenoid levels of Mature 6204 T2 seeds were extracted and quantified on an HPLC as follows. Approximately 100mg of seeds were ground in a mortar and pestle in 3ml extraction solvent (hexane/acetone/ethanol (50/25/25 v/v) with 0.3ml of an internal standard (5mg/ml β-apo-8` carotenal (dissolved in 100µl hexane), in acetonitrile/methylene chloride/methanol (50/40/10, v/v/)). The extraction solution was transferred to a new glass tube, and the remaining seed was again extracted with the 2 ml extraction solvent and pooled with first extraction solution. The extraction was repeated until no color was visible in the extraction solution. Pooled extracts were mixed by vortexing briefly, then centrifuged for approximately 5 minutes. The resulting supernatant was transfered to a new tube and dried under nitrogen gas. The dried sample was stored in the dark overnight at 4°C. The residue was resuspended in 3ml of hexane and 1 ml methanol, and 1 ml of saturated sodium chloride was added and mixed. The samples were centrifuged briefly, and the upper phase was transferred to a new tube. The remaining bottom phase was again extracted with 2 ml hexane and centrifuged. The upper phase was combined with the previous hexane phase. This was repeated until the hexane phase was colorless. The pooled hexane phases were dried under nitrogen gas, and the residue was dissolved in 2.0ml of acetonitrile/methylene chloride/methanol (50/40/10 v/v). The solution was filtered through a 0.45µm filter and colected in a brown autopsampler vial. Carotenoid concentrations were determined on a Hewlett Packard 1100 High-Performance Liquid Chromatograph (HPLC), and isocratic separation of carotenoids was performed on a Spherisorb ODS2 reverse phase C-18 (5µ) column (4.6 mm x 25cm) at 30°C. The mobile phase was 82 acetonitrile/ 10 dioxane /8 methanol (v/v) containing 150 mM ammonium acetate/ 0.1 triethylamine, with a flow rate of 1.0ml/min and a sample injection volume of 20µl (running time of 46

min). Routine detection of colored carotenoids is at 450 nm, phytoene at 280 nm, and phytofluene at 365 nm. Spectral scans for peak purity were made at 250 nm and 600 nm. Spectra of peaks at the upslope, apex, and downslope are normalized and overlaid. Superimposing spectra were taken as evidence of peak purity. The results are shown in Table 10 below, and an HPLC chromatogram is shown in Figure 17. Table 9 below describes the relevant peak retention times shown in Figure 17. Carotenoid levels are presented as μg/gFW.

Table 9.

Ret Time [min]	Area [mAU*s]	Amt/Area	Amount [ug/gFW]	Compound Name
3.500				Astaxanthin
5.428	721.34	$4.3x10^{-3}$	59.33	Lutein
5.831	169.38	$4.26x10^{-3}$	13.81	Zeaxanthin
6.533	527.83	$4.45x10^{-3}$	44.88	Canthaxanthin
7.651	553.82	$3.59x10^{-3}$	38.02	Internal Std
14.403				Echinenone
18.453	68.21	$7.02x10^{-3}$	9.16	Lycopene
22.278				Neurosporene
31.363	2966.38	$3.52x10^{-3}$	199.36	α-carotene
33.870	2854.27	$3.86x10^{-3}$	210.64	β-carotene
44.166	524.14	$1.59x10^{-2}$	158.86	Phytoene
Totals:			734.05	

Table 10. Carotenoid concentrations of canola seeds from selected T2 6204-SP30021 lines.

		Carotenoid Concentration (µg/ g FWt.)						
Sample ID	Segreg ratio	Lutein	Canth	Lycopene	a-Carotene	b-Carotene	Phytoene	Total
SP30021	homo	21	ND	ND	ND	ND	ND	21
3390-SP30021-12	homo	44	ND	9	416	578	279	1326
6204-SP30021-1	3:1	41	22	9	223	252	192	744
6204-SP30021-2	15:1	43	24	9	231	283	236	831
6204-SP30021-3	3:1	51	8	6	165	268	65	568
6204-SP30021-5	63:1	56	25	11	292	296	305	992
6204-SP30021-6	no fit	61	47	9	206	218	165	720
6204-SP30021-7	3:1	41	13	8	180	232	160	634
6204-SP30021-8	3:1	41	16	6	68	108	54	299
6204-SP30021-9	>63:1	57	39	10	233	245	245	837
6204-SP30021-10	no fit	33	9	7	165	24	103	343
6204-SP30021-11	3:1	39	7	9	198	266	145	662
6204-SP30021-12	15:1	40	15	10	212	281	172	734
6204-SP30021-13	15:1	52	44	9	207	223	247	788
6204-SP30021-15	no fit	54	20	8	205	291	160	738
6204-SP30021-21	3:1	44	11	8	142	216	126	551
6204-SP30021-24	3:1	47	9	9	149	202	89	509
6204-SP30021-25	15:1	37	33	8	235	257	243	819
6204-SP30021-28	15:1	46	11	9	225	288	123	707
6204-SP30021-29	null	18	ND	ND	ND	ND	ND	18
6204-SP30021-30	3:1	33	11	8	203	246	154	659
6204-SP30021-36	15:1	31	17	7	197	275	95	628
6204-SP30021-37	3:1	38	10	7	137	181	117	490
6204-SP30021-41	3:1	42	9	8	250	339	170	821
6204-SP30021-42	3:1	32	4	6	178	216	103	539
6204-SP30021-43	15:1	54	25	10	242	304	151	792
6204-SP30021-44	no fit	48	27	7	226	249	129	692

The initial results demonstrate that as with plants transformed to express *crt*B alone, plants expressing *crt*B and *crt*W contain significant increases in total carotenoid levels. Furthermore, the results show an increase in the levels of canthaxanthin, when compared to the levels obtained from seeds of plants transformed with *crt*B alone, as

well as nontransformed control plants. In addition, other products were also produced in plants expressing *crt*B and *crt*W. Increased levels of echineone, a reaction intermediate, as well as a putative 4-keto-α-carotene (Figure 17).

M. Carotenoid Analysis of Mature Seeds from *crtB* Transgenic *Arabidopsis* Plants

Mature 3390 T2 seeds from *Arabidopsis* were collected and carotenoid extracts were prepared and analyzed according to the method described in 2I above. These results are shown in Table 11 below. Carotenoid levels are presented as μg/gFW.

Table 11. Carotenoids of T2 Arabidopsis seeds transformed with *crtB*.

	Carotenoid concentration (μg/g FWt.)					
Sample ID	Lutein	Lycopene	α-Carotene	β-Carotene	Phytoene	Total
AT001-50 VAR	18	ND	ND	2	ND	20
3390-AT001-1	24	ND	7	20	7	58
3390-AT001-2	57	5	68	139	98	368

Initial results indicate that seeds from one line of Arabidopsis transformed with napin-*crtB* had an 18-fold increase in total carotenoid concentration. This line also demonstrate an approximately 70 fold increase in β-carotene levels (Table 11).

N. Carotenoid Analysis of Mature Seeds from *crtB* Transgenic Corn Plants

Mature 9039 T2 seeds from transgenic corn were collected and carotenoid and tocopherol extracts were prepared and analyzed according to the method described in 2I above. These results are shown in Table 12 below. Carotenoid and tocopherol levels are presented as μg/gFW.

Table 12. Carotenoid and tocopherol concentrations of T2 corn seeds transformed with *crtB*.

	Carotenoid concentration (µg/g FW)							Tocopherol concentration (µg/g FW)			
Sample ID #	Lutien	Zeaxanthin	Lycopene	a-Carotene	b-Carotene	Phytoene	Total-C	d-Tocop	g-Tocop	a-Tocop	Total-T
Control-1	7	1.92	ND	0.44	0.33	4	13	2	63	18	82
Control-2	8	2.18	ND	0.43	0.27	8	18	3	83	19	105
Control-3	8	2.16	ND	0.40	0.41	6	17	3	83	19	105
Control-4	10	2.33	ND	0.23	0.32	4	17	3	81	16	101
ZM-S4783	8	1.70	ND	0.22	1.25	82	94	5	78	14	96
ZM-S4784	5	1.53	ND	0.38	0.38	14	22	3	53	6	62
ZM-S4785	5	1.5	ND	0.13	0.75	36	44	6	113	21	140
ZM-S4789	2	0.79	ND	0.16	0.47	34	37	3	48	7	58
ZM-S4790	3	0.93	ND	0.17	0.16	6	10	3	63	11	77
ZM-S4791	4	1.42	ND	0.32	1.93	54	62	6	103	20	129
ZM-S4795	4	1.05	ND	0.39	0.57	10	17	3	64	13	80
ZM-S4796	3	0.93	ND	0.34	0.48	14	18	3	72	17	92
ZM-S4801	6	1.95	ND	0.29	0.9	16	25	4	88	19	111
ZM-S4805	7	1.65	ND	0.60	0.39	6	16	1	21	ND	23
ZM-S4814	7	1.64	ND	0.12	0.40	5	13	4	78	19	101
ZM-S4815	5	1.24	ND	0.27	0.41	7	13	4	91	19	114
ZM-S4816	2	0.92	ND	0.14	0.62	36	40	3	68	14	86
ZM-S4819	9	3.65	ND	0.19	0.81	9	23	6	52	16	75
ZM-S4820	3	0.97	ND	0.11	0.27	4	8	3	62	15	79
ZM-S4821	9	2.11	ND	0.36	0.43	4	16	2	23	18	43
ZM-S4825	3	0.87	ND	0.20	0.64	29	33	3	65	15	83
ZM-S4826	2	0.72	ND	ND	0.46	35	38	3	65	17	85

ZM-S4827	3	0.84	ND	0.08	0.54	30	34	3	74	17	94
ZM-S4830	4	1.32	ND	0.19	0.96	12	18	3	52	17	72
ZM-S4833	4	1.07	ND	0.19	0.27	2	7	1	34	15	50
ZM-S4839	4	1.21	ND	0.25	0.55	14	20	3	57	15	75
ZM-S4842	5	0.97	ND	0.26	0.19	2	9	3	69	17	89
ZM-S4847	3	0.94	ND	0.27	0.47	5	10	3	35	9	46
ZM-S4848	2	0.97	ND	0.12	0.80	19	23	2	14	10	26
ZM-S4849	6	2.85	ND	0.26	0.33	2	12	4	53	11	67
ZM-S4853	6	1.68	ND	0.30	0.47	30	38	3	61	15	79
ZM-S4854	3	1.51	ND	0.19	1.53	39	45	2	39	15	56
ZM-S4855	5	1.49	ND	0.14	0.59	33	40	4	94	20	118
ZM-S4856	3	0.95	ND	0.20	0.44	32	36	4	64	17	84
ZM-S4858	4	1.89	ND	0.15	0.21	4	11	2	34	11	47
ZM-S4861	3	1.09	ND	0.08	0.43	62	67	2	38	15	55
ZM-S4862	9	2.83	ND	0.58	0.42	7	19	4	72	15	91
ZM-S4864	5	1.68	ND	0.36	0.37	3	11	4	62	17	83
ZM-S4865	6	1.68	ND	0.37	0.32	4	12	3	76	19	98
ZM-S4866	3	0.88	ND	0.14	0.61	40	45	3	74	18	95
ZM-S4868	4	1.25	ND	0.23	0.49	29	35	2	63	21	86
ZM-S4874	8	2.55	ND	0.38	0.41	6	18	2	30	11	44
ZM-S4875	11	3.98	ND	0.34	0.53	3	20	4	76	18	97
ZM-S4878	4	1.44	ND	0.11	0.67	49	55	2	31	15	49
ZM-S4880	7	1.57	ND	0.43	0.39	3	12	3	62	17	82
ZM-S4881	6	2.45	ND	0.31	0.56	5	14	3	59	16	77
ZM-S4882	5	1.72	ND	0.42	0.46	6	14	3	55	20	78
ZM-S4884	11	2.73	ND	0.31	0.34	6	20	4	88	18	110
ZM-S4886	4	1.81	ND	0.17	0.35	38	44	4	61	15	80
ZM-S4889	8	2.46	ND	0.72	0.69	6	18	3	63	17	83

ZM-S4892	9	3.57	ND	0.29	1.21	50	64	4	74	19	97
ZM-S4893	5	1.51	ND	0.29	0.82	42	50	5	101	19	125

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Seeds from 48 transgenic corn lines were analyzed for carotenoid and tocopherol concentrations (Table 12). Total carotenoid concentration was increased up to 5 fold. The major change was phytoene (up to 15 fold increase), with a small increase in b-carotene in some lines. The total carotenoid concentration of transgenic corn seeds was only 5%-10% with respect to that of the *Brassica* seeds transformed with *crtB*. The substantial accumulation of phytoene indicates that phytoene desaturase of corn could be rate limiting. Therefore, the *crtB* effect was basically limited to phytoene biosynthesis and blocked to the later steps of the carotenoid pathway. These wild type corn seeds accumulate some zeaxanthin, but it is only about ¼ that of lutein. In order to produce more zeaxanthin in corn seeds, at least three genes (*crtB*, *crtI*, and *crtZ*)may be required.

O. Expression of *Maize* phytoene synthase in *Arabidopsis*

Mature 9061 T2 seeds from *Arabidopsis* were collected and carotenoid extracts were prepared and analyzed according to the method described in 2I above. These results are shown in Table 13 below and in Figure 18. Carotenoid levels are presented as μg/gFW.

Table 13: Results of Carotenoid composition determined by HPLC on *A.t.* 9061 lines

Strain ID	Lutein	Lycopene	α-Carotene	β-Carotene	Phytoene	Total-Major-C	%phytoene
AT001	27	0	0	8	0	34	0
9061-AT001-1	36	4	20	82	15	157	9.7
9061-AT001-2	61	6	115	295	115	591	19.3
9061-AT001-3	29	4	22	82	24	160	15.0
9061-AT001-4	68	5	78	251	60	461	12.9
9061-	42	6	62	163	63	336	18.7

AT001-5							
9061-AT001-6	46	4	59	167	60	337	17.8
9061-AT001-7	31	4	17	61	6	119	4.9
9061-AT001-8	58	7	80	230	85	460	18.4
9061-AT001-9	27	4	24	57	25	138	17.9
9061-AT001-10	64	6	82	243	78	473	16.5
9061-AT001-11	52	6	79	225	72	434	16.4
9061-AT001-12	53	5	74	222	69	424	16.3
9061-AT001-13	43	5	62	217	55	382	14.3
9061-AT001-14	49	5	73	185	83	395	20.9
9061-AT001-15	26	4	13	60	9	112	8.4
9061-AT001-16	33	5	52	157	50	297	16.7
9061-AT001-17	69	6	93	293	86	547	15.7
9061-AT001-18	41	5	46	164	24	280	8.5
9061-AT001-19	55	6	85	206	103	455	22.7
9061-AT001-20	71	6	116	244	125	562	22.3
9061-	61	5	88	226	89	469	18.9

AT001-21							
9061-AT001-22	49	6	66	198	76	395	19.2
9061-AT001-23	44	4	44	167	22	282	7.8
9061-AT001-24	59	6	70	219	57	411	13.9
9061-AT001-25	58	6	65	228	70	426	16.4
9061-AT001-26	57	6	81	212	80	436	18.2
9061-AT001-27	17	0	8	18	0	44	0
9061-AT001-28	55	5	76	176	65	378	17.2

Transgenic *Arabidopsis* expressing the maize phytoene synthase demonstrate an increase in total carotenoid levels. There are 11 lines of pCGN 9061 that have total carotenoids in excess of 400 ppm. This is higher than the highest pCGN 3390 line which is 381ppm. There are three *Arabidopsis* lines containing the maize phytoene synthase with total carotenoids between 550ppm and 590ppm.

Also, transgenic *Arabidopsis* lines containing the maize phytoene synthase produce less phytoene as a percentage of total carotenoids. The pCGN 9061 lines that accumulate the most carotenoids have only 16%-22% phytoene. The pCGN 3390 lines that accumulate the most carotenoids have a percent phytoene range from 25%-31% phytoene. The β-carotene is also significantly increased (Figure 18).

Example 3 Crosses of *crtB* Plants

A. Transgenic Oil Traits

To evaluate the high oleic trait of the napin-crtB transgenic plants in conjunction with expression of other oils traits, crosses off 3390-1-6-8 with a mangosteen thioesterase (5266) and a nutmeg thioesterase (3854; see WO 96/23892) were made. Crosses were also made with two low linoleic (LPOO4 and LP30108) varieties. Half-seed analyses of carotenoids and fatty acid composition were conducted on the segregating seeds, and the average of the half seed values are shown below in Tables 13 and 14.

TABLE 13

Carotenoid Levels in Half Seeds Resulting from 3390 Crosses

Cross	Lutein	Lycopene	α-Carotene	ß-Carotene	Total
F1 3390-SP001-1-6-8 x SP30021	21.6	26.2	271.5	413.1	732.4
F1 3390-SP001-1-6-8 x 5266-SP30021-5-26	18.0	21.7	187.9	284.1	511.7
F1 3390-SP001-1-6-8 x 5266-SP30021-35-2	16.2	22.1	223.0	318.4	579.7
F1 3390-SP001-1-6-8 x 5266-SP30021-35-12	19.5	22.9	196.8	312.8	552.0
F1 3390-SP001-1-6-8 x LP30108-19	23.7	22.7	213.4	355.0	614.8
F1 LP30108-19 x F1 3390-SP001-1-6-8	16.4	19.6	156.7	224.5	417.2

TABLE 14

Fatty Acid Composition in Half Seeds Resulting from 3390 Crosses

STRAIN_ID	%14:0	%16:0	%18:0	%18:1	%18:2	%18:3	%20:0
(3390-SP001-1-6-8 X SP30021)	0.05	3.55	1.70	74.78	11.29	5.71	0.73

(3390-SP001-1-6-8 X 5266-SP30021-35-12)	0.06	3.84	11.37	62.86	11.06	5.08	3.38
(3390-SP001-1-6-8 X 5266-SP30021-35-2)	0.06	3.68	11.27	64.80	9.81	5.16	3.04
3390-SPOO1-1-6-8 X 5266-SP30021-5-26	0.06	3.66	15.36	60.78	9.30	4.77	3.87
(3390-SP001-1-6-1 X 3854-SP30021-20-3)	2.69	9.80	3.65	64.62	9.72	4.57	1.51
(3390-SP001-1-6-1 X 3854-SP30021-20-1)	6.14	16.35	5.12	54.91	8.23	4.23	2.03
(3390-SP001-1-6-1 X 5266-LP004-2-31)	0.07	3.82	11.67	64.52	11.46	3.14	3.08
(3390-SP001-1-6-8 X LP30108-19)	0.05	3.80	1.44	73.66	14.02	3.93	0.67
(LP30108-19 X 3390-SP001-1-6-8)	0.04	3.31	1.79	79.69	9.26	2.97	0.75
SPOO1-4-10	0.07	4.44	0.99	56.06	21.79	14.31	0.44
3390-SPOO1-1-6-8	0.04	3.46	1.44	77.26	9.30	5.71	0.63

As the above results demonstrate, a dramatic increase (100 to 200 fold) in α- and β-carotene as well as a 60 fold increase in total carotenoids may be obtained by transformation of plants for expression of an early carotenoid biosynthesis gene under the regulatory control of promoter preferentially expressed in plant seed tissue. This increase in flux primes the pathway for the production of specialty products as described above, and also results in increased production of α-tocopherol (Vitamin E).

Furthermore, it is evident that the fatty acid composition can also be altered in the transgenic plant seeds. In this manner, seeds can be used to produce novel products, to provide for production of particular carotenoids, to provide high oleic oils, and the like.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

IN THE CLAIMS

What is claimed is:

1. A method for altering the carotenoid content in seed of a host corn plant, comprising:

transforming cells of a host corn plant with a construct comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA sequence derived from a carotenoid biosynthesis gene coding region, and a transcriptional termination region,

producing a transformed host corn plant from said transformed cells, and

growing said transformed host corn plant or progeny thereof containing said construct under conditions whereby seed is produced having an altered carotenoid content.

2. The method according to Claim 1, wherein said altered carotenoid content is increased.

3. The method according to Claim 1, wherein said carotenoid biosynthesis gene is an early carotenoid biosynthesis gene.

4. The method according to Claim 3, wherein said early carotenoid biosynthesis gene is selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

5. The method according to Claim 1, further comprising introducing into a host corn cell a second construct comprising as operably linked components, a promoter functional in a corn seed cell, a second carotenoid biosynthesis gene, and a transcriptional termination region functional in a corn seed cell.

6. The method according to Claim 5, wherein said second carotenoid biosynthesis gene is selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, isopentenyl diphosphate isomerase, β-carotene hydroxylase, the astaxanthin biosynthesis enzyme encoded by *crt*W, and ε-hydroxylase, or wherein said secondary gene results in inhibition of transcription of an endogenous plant gene encoding lycopene ε-cyclase, lycopene β-cyclase or phytoene desaturase.

7. A method for the alteration of carotenoid content in the endosperm of a host plant, comprising:

transforming cells of a host plant with a construct comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed endosperm, a plastid transit peptide, a DNA sequence derived from a carotenoid biosynthesis gene coding region, and a transcriptional termination region,

producing a transformed host corn plant from said transformed cells, and

growing said transformed host corn plant or progeny thereof containing said construct under conditions whereby seed is produced having an endosperm having an altered carotenoid content.

8. The method according to Claim 7, wherein said carotenoid content is increased.

9. The method according to Claim 7, wherein said carotenoid biosynthesis gene is an early carotenoid biosynthesis gene.

10. The method according to Claim 7, wherein said early carotenoid biosynthesis gene is selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

11. The method according to Claim 7, further comprising introducing into a host corn cell a second construct comprising as operably linked components, a promoter functional in a corn seed cell, a second carotenoid biosynthesis gene, and a transcriptional termination region functional in a corn seed cell.

12. The method according to Claim 11, wherein said second carotenoid biosynthesis gene is selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, isopentenyl diphosphate isomerase, β-carotene hydroxylase, the astaxanthin biosynthesis enzyme encoded by *crt*W, and ε-hydroxylase, or wherein said secondary gene results in inhibition of transcription of an endogenous plant gene encoding lycopene ε-cyclase, lycopene β-cyclase or phytoene desaturase.

13. A method for screening transformed corn seeds or transformed endosperms, comprising:

transforming a host corn plant with an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a transit peptide, a DNA coding sequence of at least one carotenoid biosynthesis gene, and a transcriptional termination region, and

selecting said transformed seeds or transformed endosperms exhibiting a yellow, orange or red color.

14. The method according to Claim 13, wherein said early carotenoid biosynthesis gene encodes an enzyme selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

15. The method according to Claim 13, further comprising introducing into a host corn cell a second construct comprising as operably linked components, a promoter

functional in a corn seed cell, a second carotenoid biosynthesis gene, and a transcriptional termination region functional in a corn seed cell.

16. The method according to Claim 15, wherein said second carotenoid biosynthesis gene is selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, isopentenyl diphosphate isomerase, β-carotene hydroxylase, the astaxanthin biosynthesis enzyme encoded by *crt*W, and ε-hydroxylase, or wherein said secondary gene results in inhibition of transcription of an endogenous plant gene encoding lycopene ε-cyclase, lycopene β-cyclase or phytoene desaturase.

17. A nucleic acid sequence encoding a phytoene synthase from corn.

18. The nucleic acid sequence according to Claim 17, wherein said sequence is the sequence set forth in Figure 19.

19. A method for altering carotenoid composition in a seed from a host plant, said method comprising transforming said host plant with a construct comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a nucleic acid sequence encoding a carotenoid biosynthesis gene from eukaryotic source, and a transcriptional termination region.

20. The method according to Claim 19, wherein said carotenoid biosynthesis gene is phytoene synthase.

21. The method according to Claim 19, wherein said carotenoid biosynthesis gene is from corn.

22. The method according to Claim 19, wherein said carotenoid biosynthesis gene is encoded by the nucleic acid sequence set forth in Figure 19.

SEQUENCE LISTING

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<120> Methods for Producing Carotenoid Compounds, and Speciality Oils in Plant Seeds

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Glu Glu His Asp His Ala Leu Glu Lys Asn Asp Leu Tyr Gly Leu Val
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gctgcttggt tggctttgca cgttcacgct ttgtggttct tggacgctgc tgctcaccca 300

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gatccagttt ccttgttgac ctgtttccac ttcggtggtt accaccacga gcaccacttg 840

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ta 902

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Met Thr Gly Phe Pro Val Lys Lys Val Asn Thr Asp Ile Thr Ser Ile
        35                  40                  45

Thr Ser Asn Gly Gly Arg Val Lys Cys Met Ser Ala His Ala Leu Pro
    50                  55                  60

Lys Ala Asp Leu Thr Ala Thr Ser Leu Ile Val Ser Gly Gly Ile Ile
65                  70                  75                  80

Ala Ala Trp Leu Ala Leu His Val His Ala Leu Trp Phe Leu Asp Ala
                85                  90                  95

Ala Ala His Pro Ile Leu Ala Ile Ala Asn Phe Leu Gly Leu Asn Trp
            100                 105                 110

Leu Ser Val Gly Leu Phe Ile Ile Ala His Asp Ala Met His Gly Ser
        115                 120                 125

Val Val Pro Gly Arg Pro Arg Ala Asn Ala Ala Met Gly Gln Leu Val
    130                 135                 140

Leu Trp Leu Tyr Ala Gly Phe Ser Trp Arg Lys Met Ile Val Lys His
145                 150                 155                 160

Met Ala His His Arg His Ala Gly Thr Asp Asp Asp Pro Asp Phe Asp
                165                 170                 175

His Gly Gly Pro Val Arg Trp Tyr Ala Arg Phe Ile Gly Thr Tyr Phe
            180                 185                 190

Gly Trp Arg Glu Gly Leu Leu Leu Pro Val Ile Val Thr Val Tyr Ala
        195                 200                 205

Leu Ile Leu Gly Asp Arg Trp Met Tyr Val Val Phe Trp Pro Leu Pro
    210                 215                 220

Ser Ile Leu Ala Ser Ile Gln Leu Phe Val Phe Gly Thr Trp Leu Pro
225                 230                 235                 240

His Arg Pro Gly His Asp Ala Phe Pro Asp Arg His Asn Ala Arg Ser
                245                 250                 255

Ser Arg Ile Ser Asp Pro Val Ser Leu Leu Thr Cys Phe His Phe Gly
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Gly Tyr His His Glu His His Leu His Pro Thr Val Pro Trp Trp Arg
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Leu Pro Ser Thr Arg Thr Lys Gly Asp Thr Ala
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290 295

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tcaagacgaa gaggccggcg gcgcgccggt ggatgccctg ctcgctcctt ggcctccacc 180

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cacgcaacgg gctcaaggaa gcctacgacc gctgcggcga gatctgtgag gagtatgcca 420

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tcgacgagct ctacatgtac tgctactatg ttgctggaac tgtcgggtta atgagcgtac 780

ctgtgatggg catcgcaacc gagtctaaag caacaactga aagcgtatac agtgctgcct 840

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            20                  25                  30

Thr Lys Arg Pro Ala Ala Arg Arg Trp Met Pro Cys Ser Leu Leu Gly
        35                  40                  45

Leu His Pro Trp Glu Ala Gly Arg Pro Ser Pro Ala Val Tyr Ser Ser
    50                  55                  60

Leu Ala Val Asn Pro Ala Gly Glu Ala Val Val Ser Ser Glu Gln Lys
65                  70                  75                  80

Val Tyr Asp Val Val Leu Lys Gln Ala Ala Leu Leu Lys Arg Gln Leu
                85                  90                  95

Arg Thr Pro Val Leu Asp Ala Arg Pro Gln Asp Met Asp Met Pro Arg
            100                 105                 110

Asn Gly Leu Lys Glu Ala Tyr Asp Arg Cys Gly Glu Ile Cys Glu Glu
        115                 120                 125

Tyr Ala Lys Thr Phe Tyr Leu Gly Thr Met Leu Met Thr Glu Glu Arg
    130                 135                 140

Arg Arg Ala Ile Trp Ala Ile Tyr Val Trp Cys Arg Arg Thr Asp Glu
145                 150                 155                 160

Leu Val Asp Gly Pro Asn Ala Asn Tyr Ile Thr Pro Thr Ala Leu Asp
                165                 170                 175

Arg Trp Glu Lys Arg Leu Glu Asp Leu Phe Thr Gly Arg Pro Tyr Asp
            180                 185                 190

Met Leu Asp Ala Ala Leu Ser Asp Thr Ile Ser Arg Phe Pro Ile Asp
        195                 200                 205

Ile Gln Pro Phe Arg Asp Met Ile Glu Gly Met Arg Ser Asp Leu Arg
    210                 215                 220

Lys Thr Arg Tyr Asn Asn Phe Asp Glu Leu Tyr Met Tyr Cys Tyr Tyr
225                 230                 235                 240

Val Ala Gly Thr Val Gly Leu Met Ser Val Pro Val Met Gly Ile Ala
                245                 250                 255
```

```
Thr Glu Ser Lys Ala Thr Thr Glu Ser Val Tyr Ser Ala Ala Leu Ala
            260                 265                 270

Leu Gly Ile Ala Asn Gln Leu Thr Asn Ile Leu Arg Asp Val Gly Glu
        275                 280                 285

Asp Ala Arg Arg Gly Arg Ile Tyr Leu Pro Gln Asp Glu Leu Ala Gln
    290                 295                 300

Ala Gly Leu Ser Asp Glu Asp Ile Phe Lys Gly Val Val Thr Asn Arg
305                 310                 315                 320

Trp Arg Asn Phe Met Lys Arg Gln Ile Lys Arg Ala Arg Met Phe Phe
                325                 330                 335

Glu Glu Ala Glu Arg Gly Val Asn Glu Leu Ser Gln Ala Ser Arg Trp
            340                 345                 350

Pro Val Trp Ala Ser Leu Leu Leu Tyr Arg Gln Ile Leu Asp Glu Ile
        355                 360                 365

Glu Ala Asn Asp Tyr Asn Asn Phe Thr Lys Arg Ala Tyr Val Gly Lys
    370                 375                 380

Gly Lys Lys Leu Leu Ala Leu Pro Val Ala Tyr Gly Lys Ser Leu Leu
385                 390                 395                 400

Leu Pro Cys Ser Leu Arg Asn Gly Gln Thr Xaa Pro Pro Glu Lys Leu
                405                 410                 415

Gln Asp Pro Pro Arg Asp Xaa Arg Ala
            420                 425
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BglII
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TTCCATTACA AGCAATGGTG GAAGAGTAAA GTGCATGAAT AATCCGTCGT TACTCAATCA 240

TGCGGTCGAA ACGATGGCAG TTGGCTCGAA AAGTTTTGCG ACAGCCTCAA AGTTATTTGA 300

TGCAAAAACC CGGCGCAGCG TACTGATGCT CTACGCCTGG TGCCGCCATT GTGACGATGT 360

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ACGTCTGATG CAACTTGAGA TGAAAACGCG CCAGGCCTAT GCAGGATCGC AGATGCACGA 480

ACCGGCGTTT GCGGCTTTTC AGGAAGTGGC TATGGCTCAT GATATCGCCC CGGCTTACGC 540

GTTTGATCAT CTGGAAGGCT TCGCCATGGA TGTACGCGAA GCGCAATACA GCCAACTGGA 600

TGATACGCTG CGCTATTGCT ATCACGTTGC AGGCGTTGTC GGCTTGATGA TGGCGCAAAT 660

FIGURE 1

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GGCAAGCTGG CTGGAGCATG AAGGTCTGAA CAAAGAGAAT TATGCGGCAC CTGAAAACCG    840

TCAGGCGCTG AGCCGTATCG CCCGTCGTTT GGTGCAGGAA GCAGAACCTT ACTATTTGTC    900

TGCCACAGCC GGCCTGGCAG GGTTGCCCCT GCGTTCCGCC TGGGCAATCG CTACGGCGAA    960

GCAGGTTTAC CGGAAAATAG GTGTCAAAGT TGAACAGGCC GGTCAGCAAG CCTGGGATCA   1020

GCGGCAGTCA ACGACCACGC CCGAAAAATT AACGCTGCTG CTGGCCGCCT CTGGTCAGGC   1080

CCTTACTTCC CGGATGCGGG CTCATCCTCC CCGCCCTGCG CATCTCTGGC AGCGCCCGCT   1140

CTAGCGCCAT GTCTTTCCCG GAGCGTCCGA ATTATCGATG AATTCGAGCT CGGTACCCGG   1200

BamHI
|
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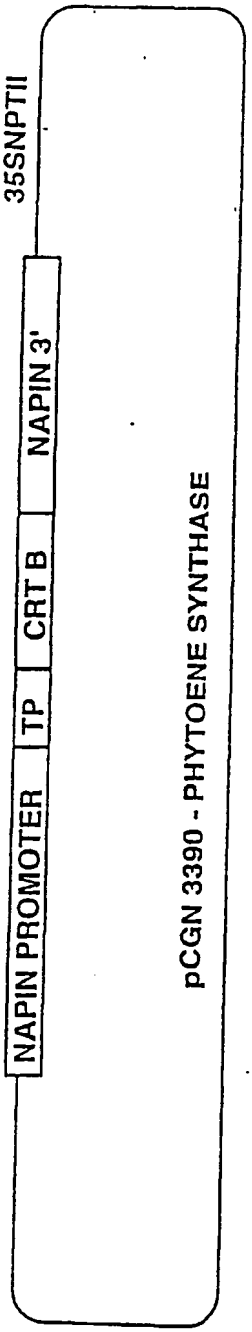
FIGURE 1



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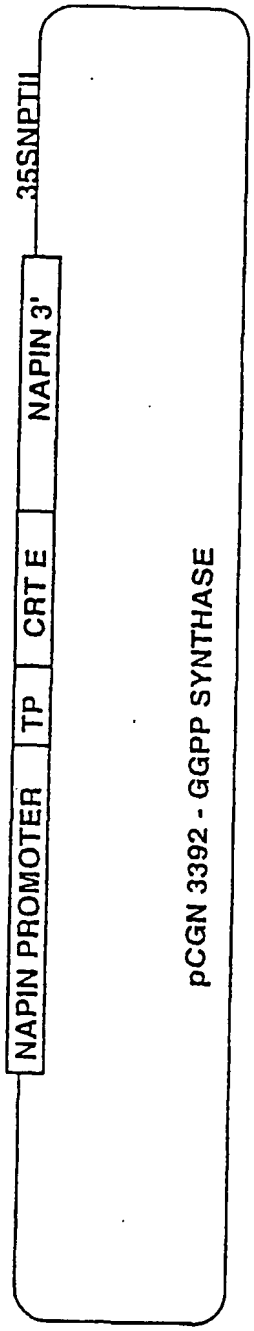

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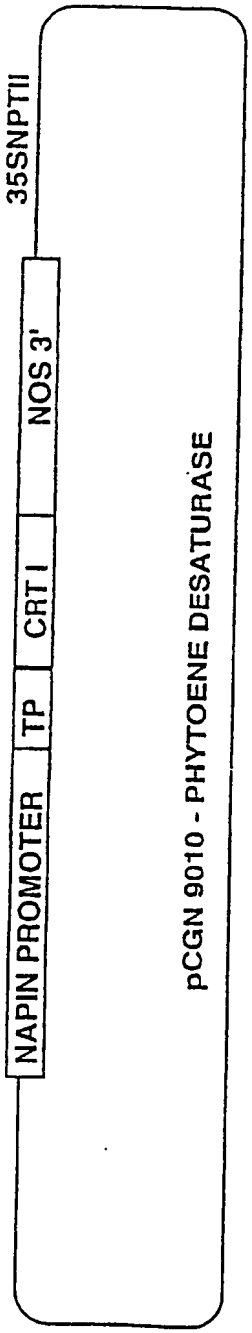

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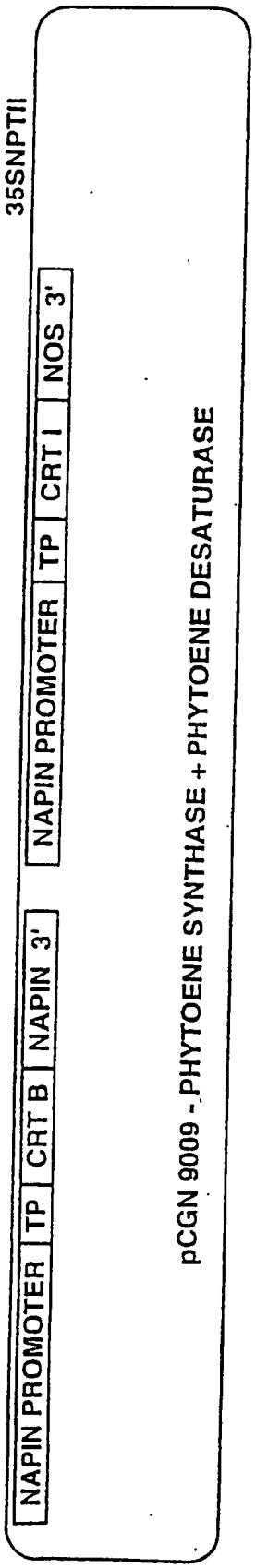

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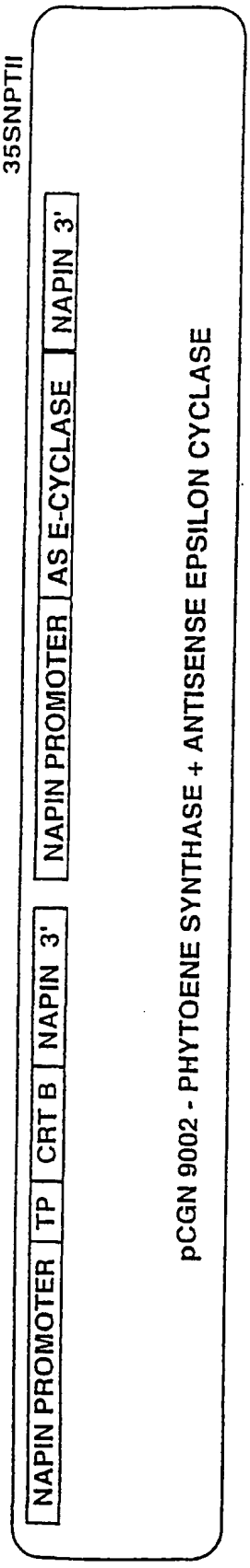

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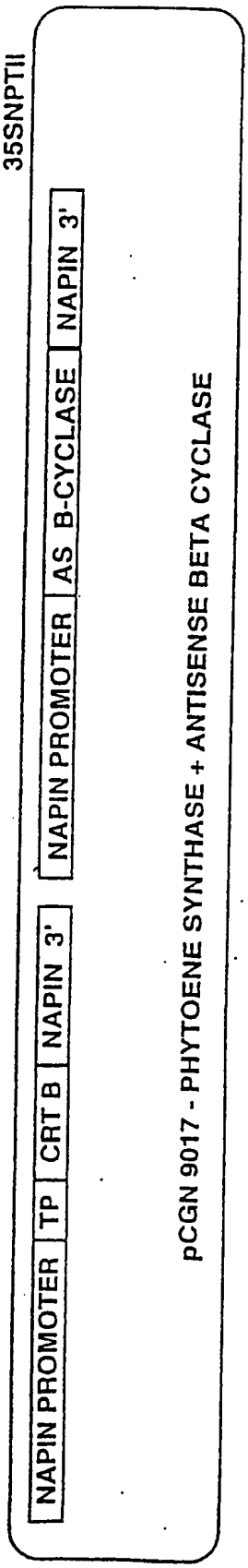

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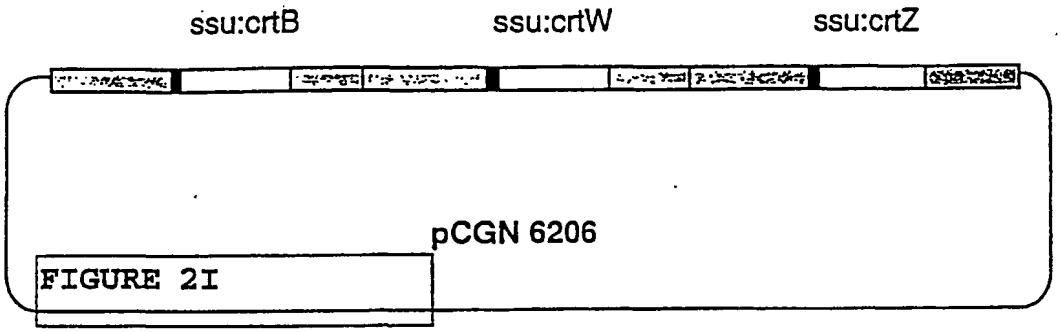
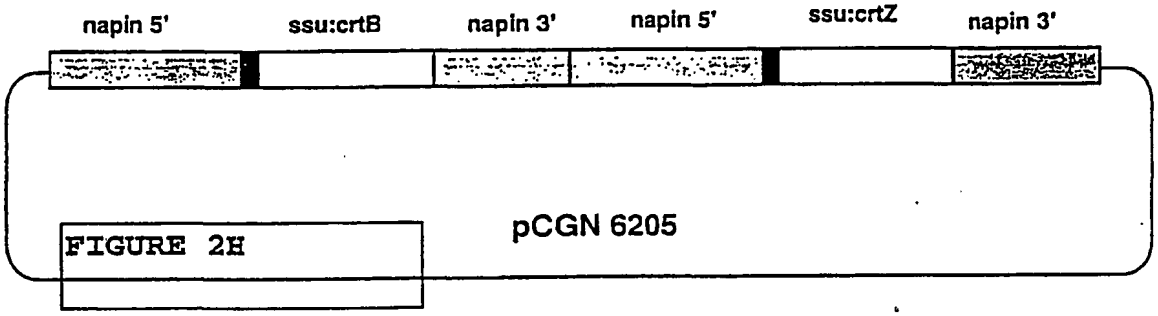


FIGURE 2I



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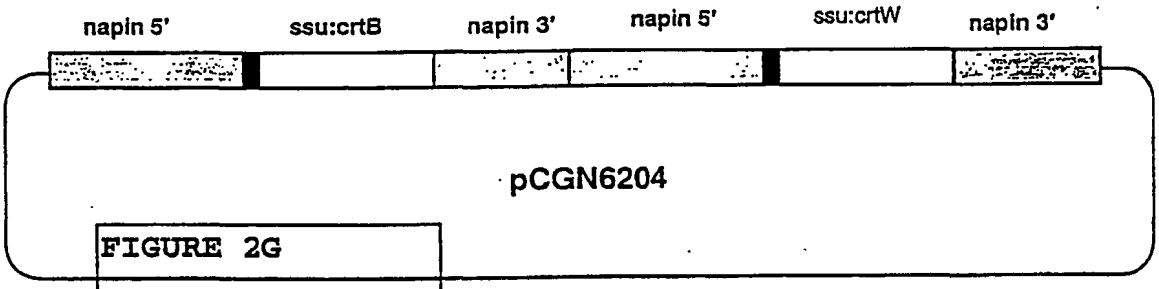
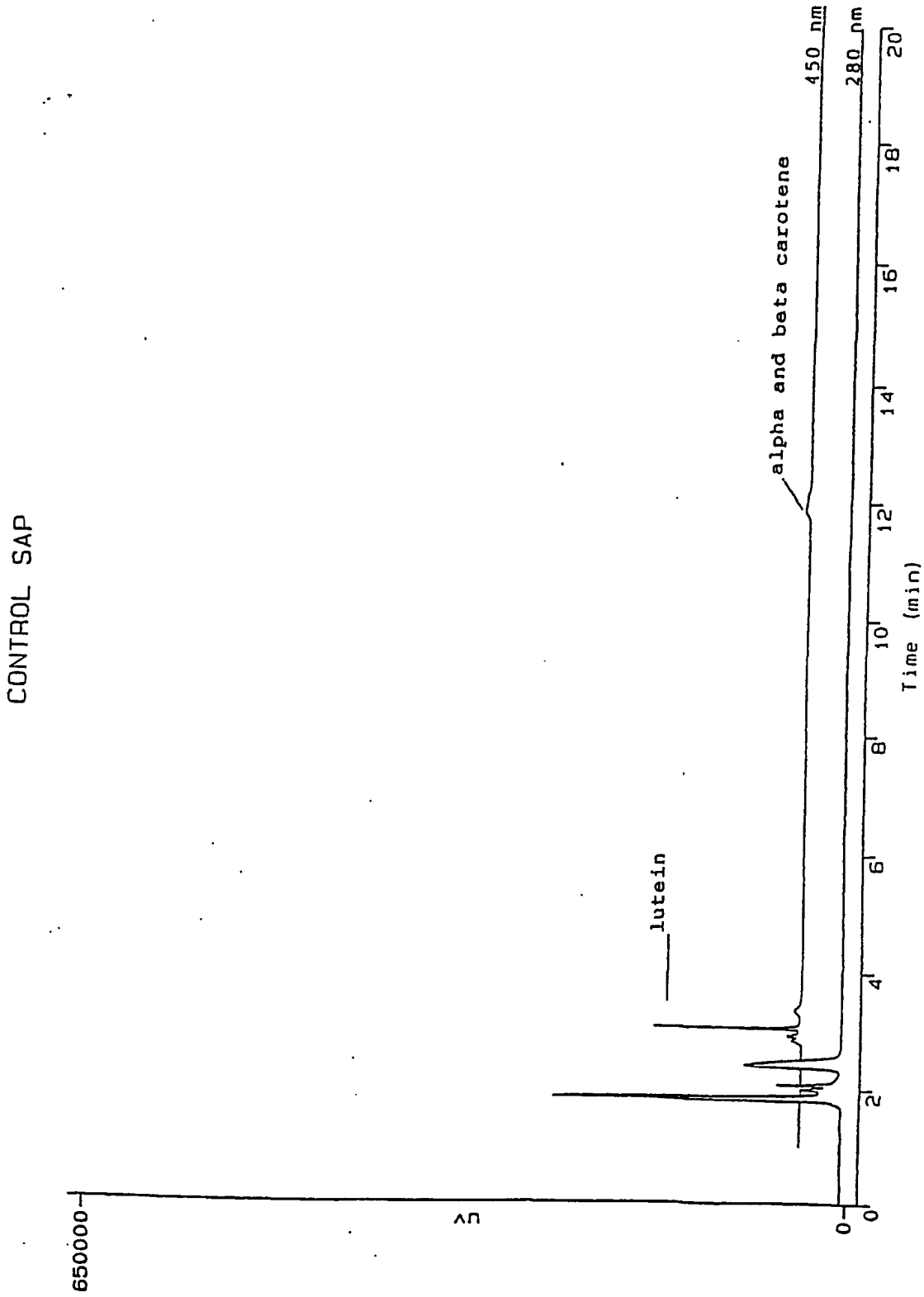


FIGURE 2G

napin cassette | plastid targetting | crt B, crt Z or crt W as indicated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650000
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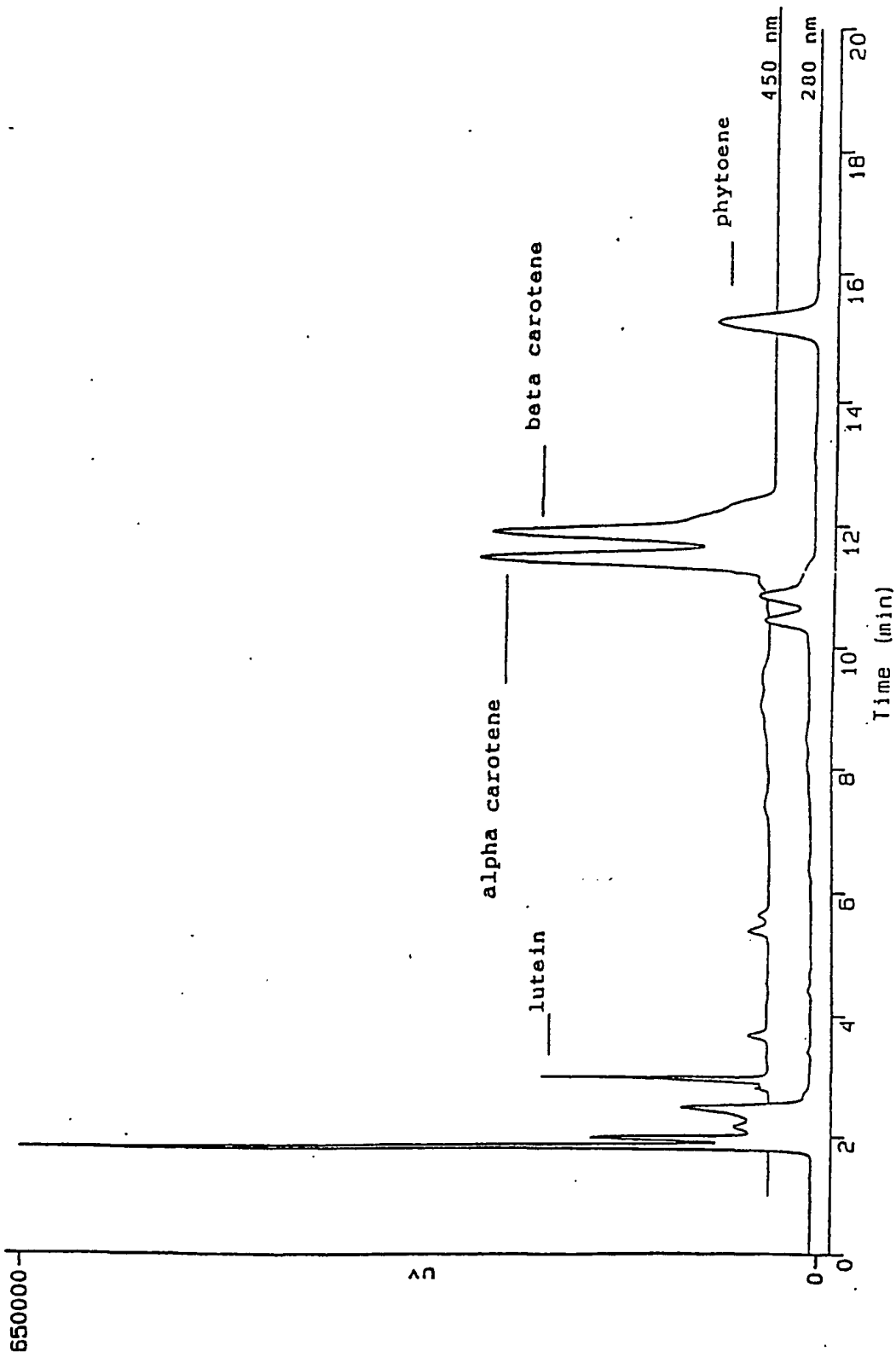
UV
450 nm
280 nm
lutein
alpha and beta carotene
0
2
4
6
8
10
12
14
16
18
20
Time (min)

FIGURE 3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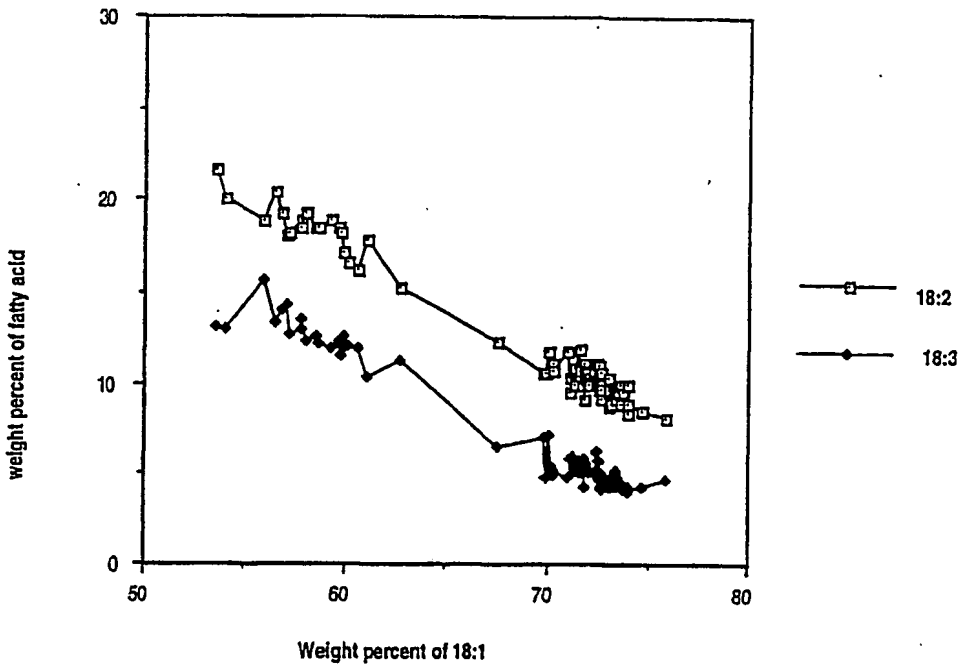
18
20
Time (min)
450 nm
280 nm
phytoene
beta carotene
alpha carotene
lutein

FIGURE 4


18:1 vs 18:2 and 18:3 in 3390s
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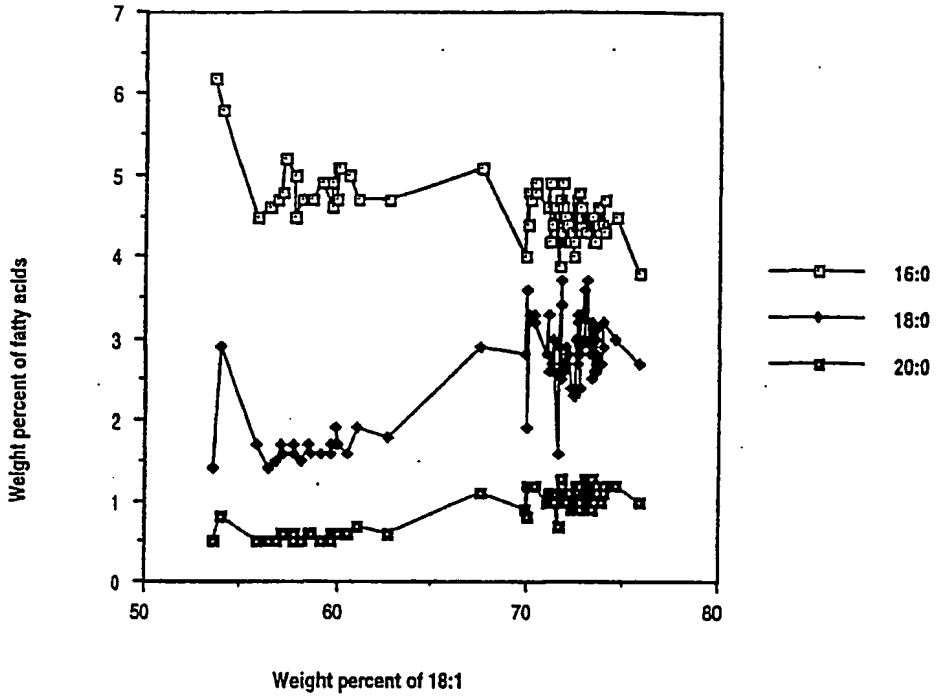
10
0
50
60
70
80
weight percent of fatty acid
Weight percent of 18:1
18:2
18:3

FIGURE 5


18:1 vs 16:0, 18:0 & 20:0 in 3390s
Weight percent of fatty acids
Weight percent of 18:1
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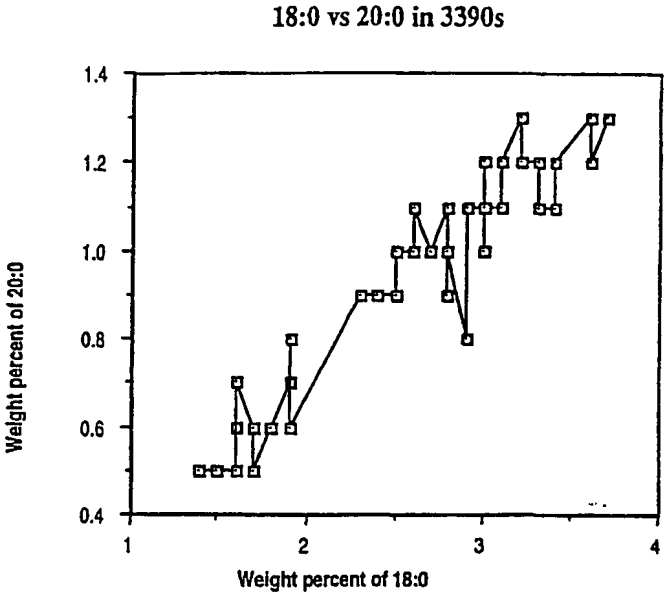
3
4
5
6
7
50
60
70
80
16:0
18:0
20:0

FIGURE 6


18:0 vs 20:0 in 3390s
Weight percent of 20:0
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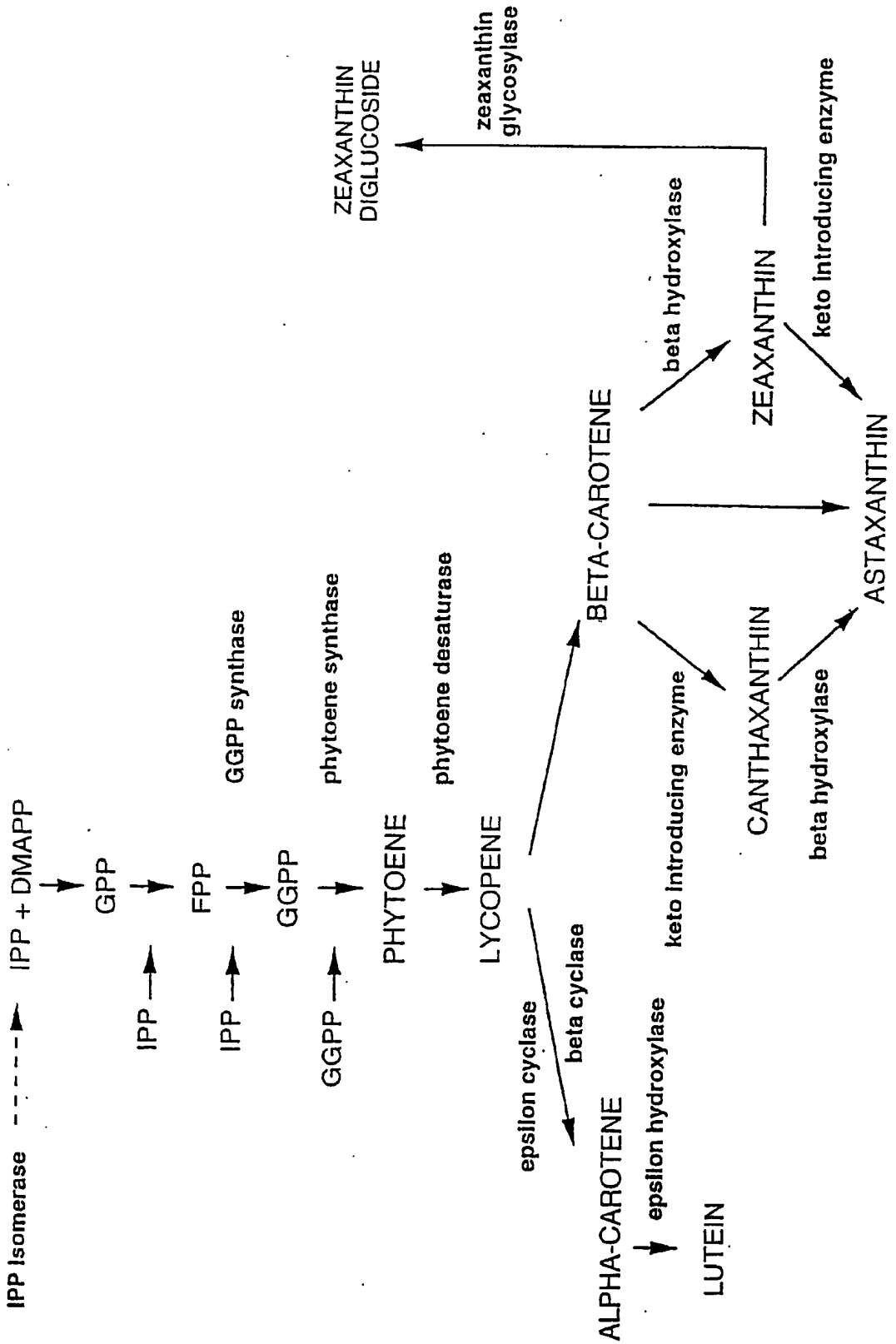
1.2
1.0
0.8
0.6
0.4
1
2
3
4
Weight percent of 18:0

FIGURE 7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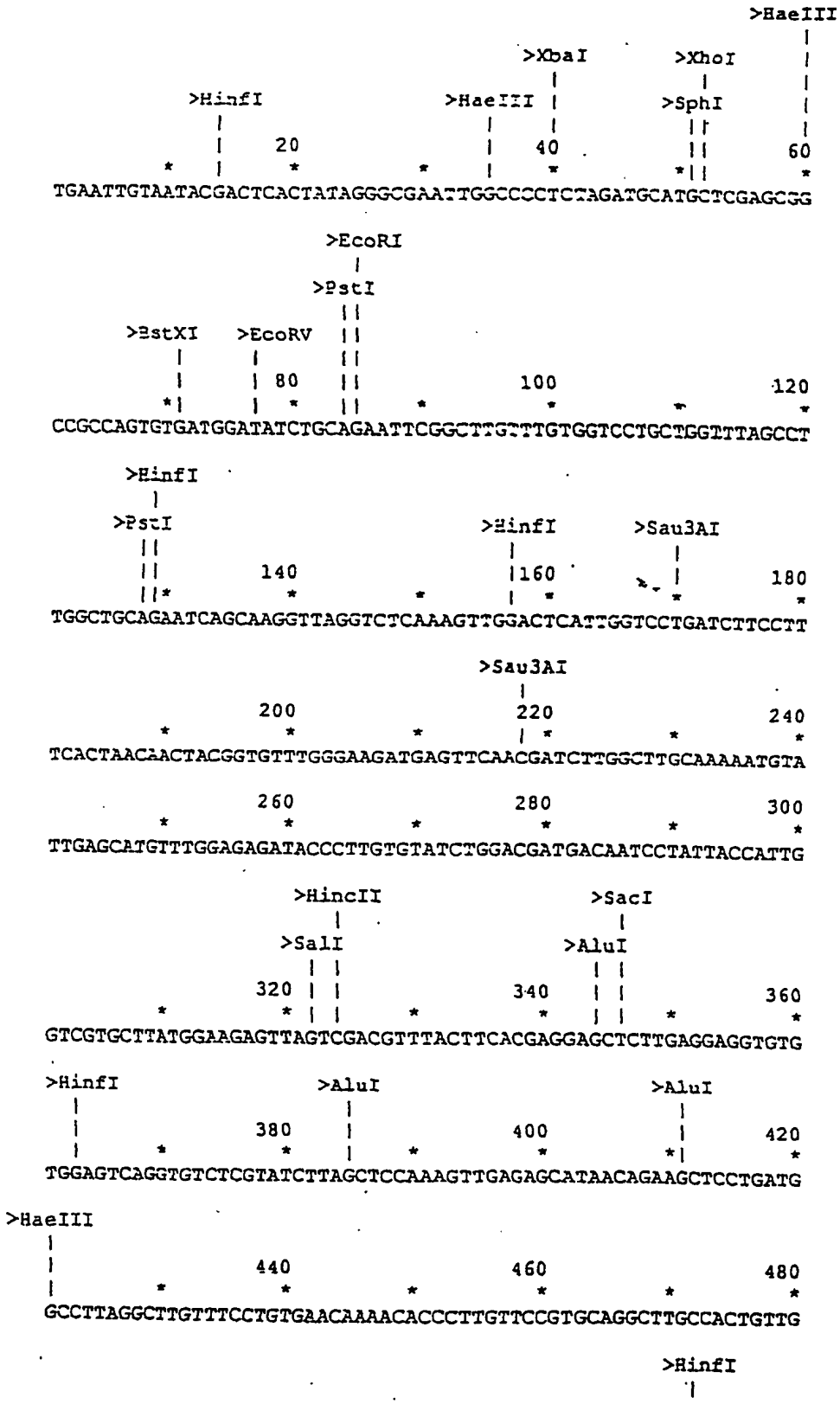
IPP + DMAPP
GPP
IPP
FPP
IPP
GGPP synthase
GGPP
GGPP
phytoene synthase
PHYTOENE
phytoene desaturase
LYCOPENE
epsilon cyclase
beta cyclase
ALPHA-CAROTENE
epsilon hydroxylase
LUTEIN
keto introducing enzyme
BETA-CAROTENE
beta hydroxylase
ZEAXANTHIN
zeaxanthin glycosylase
ZEAXANTHIN DIGLUCOSIDE
CANTHAXANTHIN
beta hydroxylase
keto introducing enzyme
ASTAXANTHIN

FIGURE 8
1/1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100
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>PstI
>HinfI
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>SacI
>SalI
>AluI
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340
360
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>AluI
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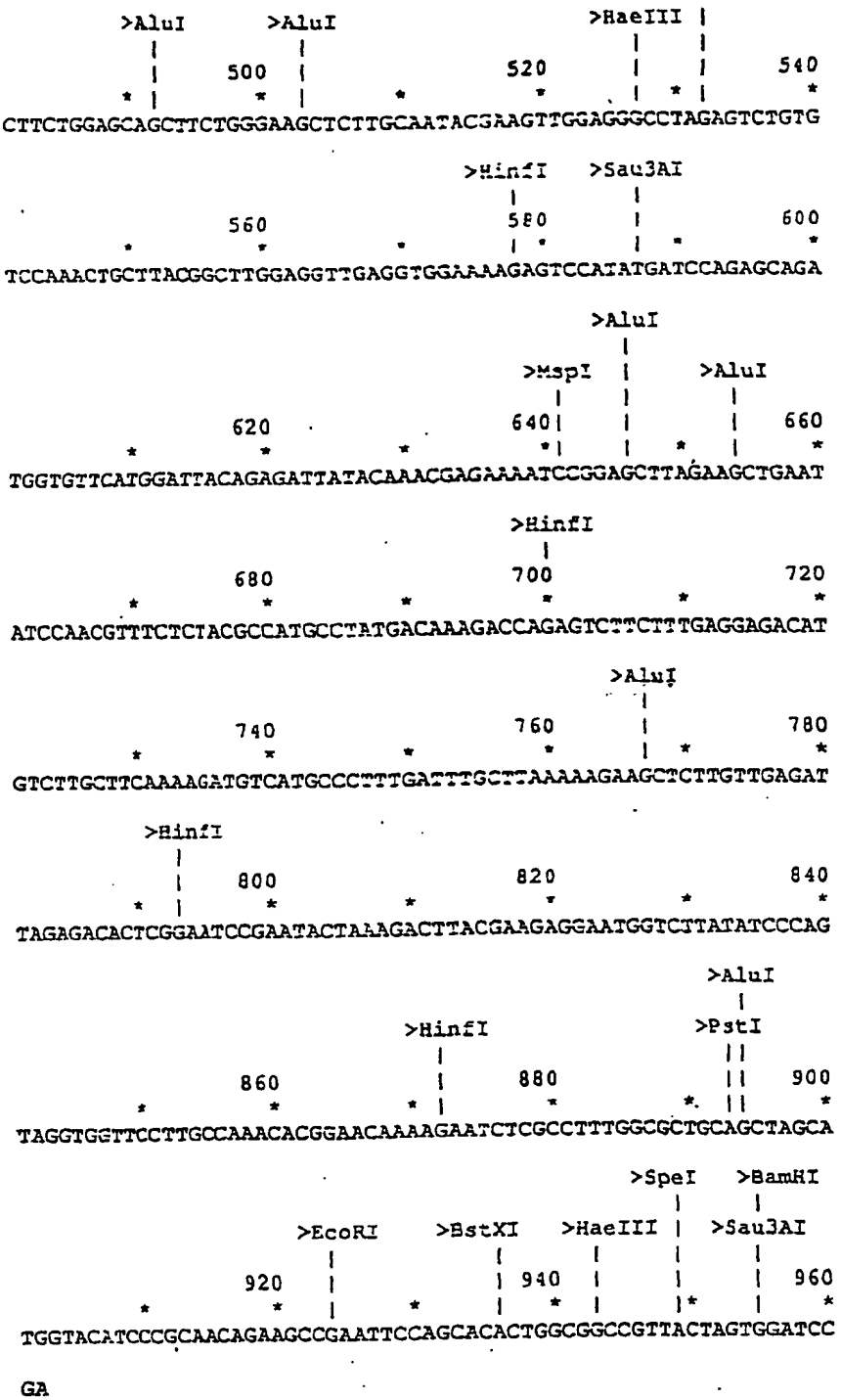
FIGURE 9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520
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>PstI
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>BamHI
>EcoRI
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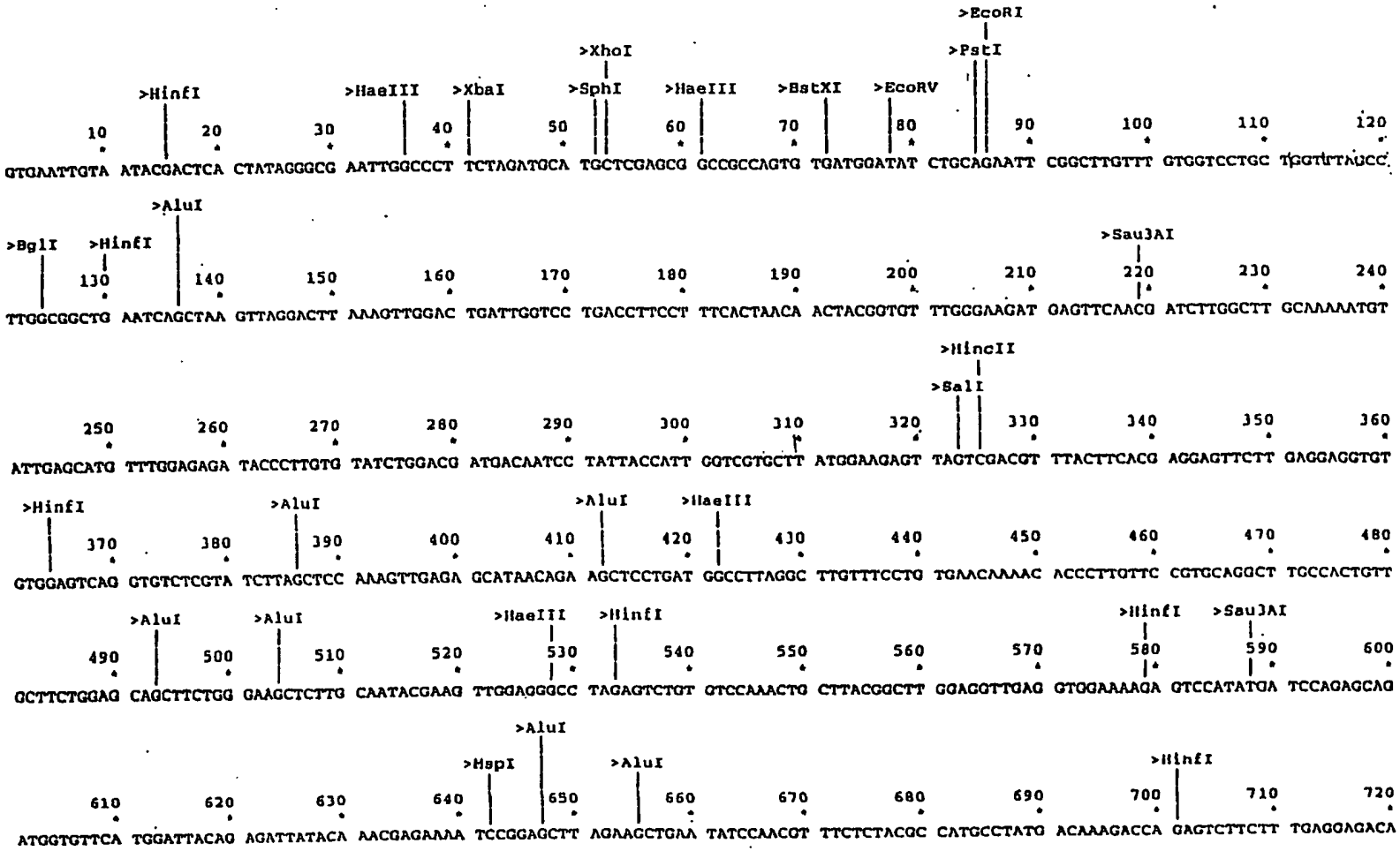
FIGURE 9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>HinfI
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>AluI
>AluI
>HaeIII
>HinfI
>HinfI
>Sau3AI
490 500 510 520 530 540 550 560 570 580 590 600
GCTTCTGGAG CAGCTTCTGG GAAGCTCTTG CAATACGAAG TTGGAGGGCC TAGAGTCTGT GTCCAAACTG CTTACGGCTT GGAGGTTGAG GTGGAAAAGA GTCCATATGA TCCAGAGCAG
>AluI
>HspI
>AluI
>HinfI
610 620 630 640 650 660 670 680 690 700 710 720
ATGGTGTTCA TGGATTACAG AGATTATACA AACGAGAAAA TCCGGAGCTT AGAAGCTGAA TATCCAACGT TTCTCTACGC CATGCCTATG ACAAAGACCA GAGTCTTCTT TGAGGAGACA

FIGURE 10

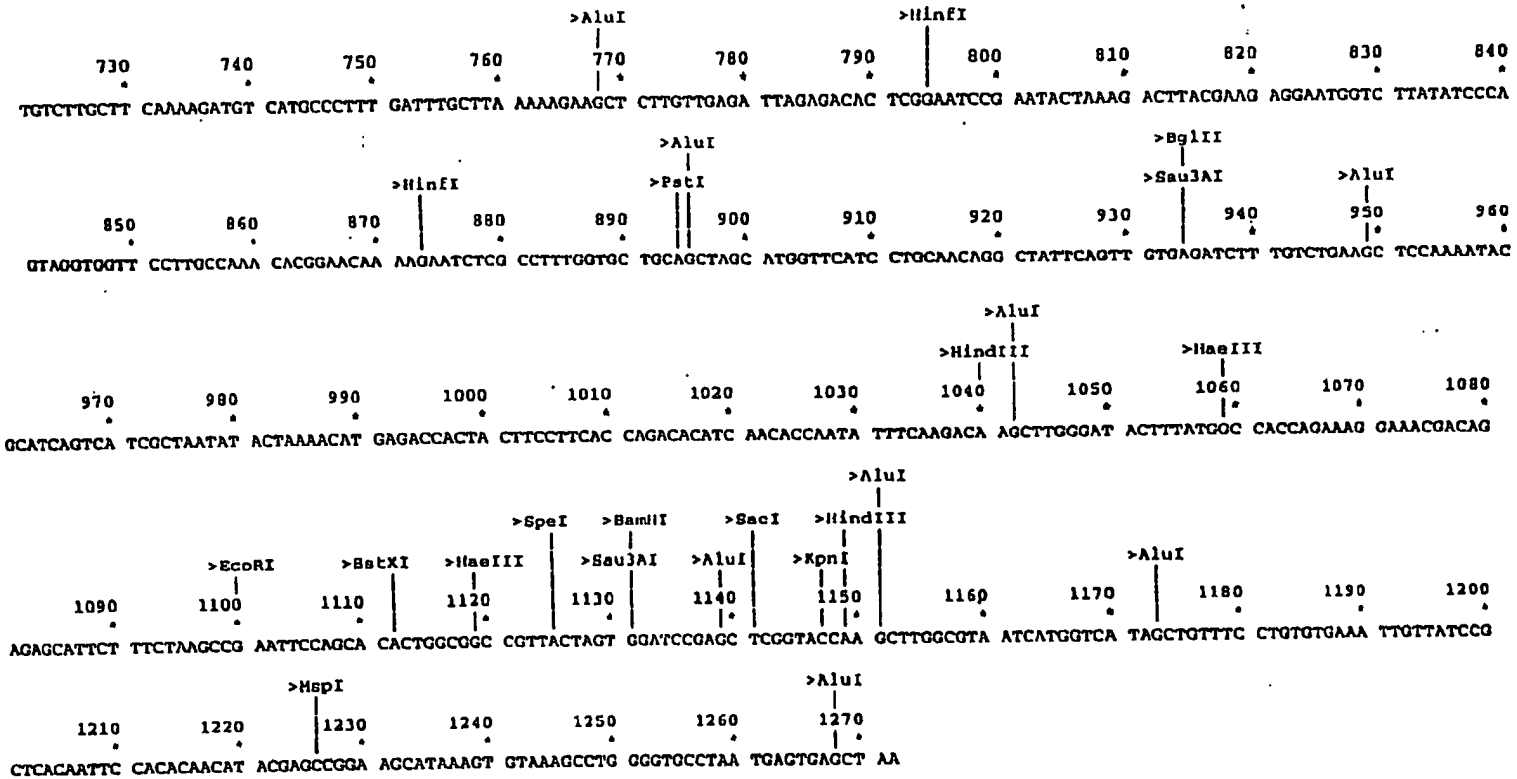

>AluI
>HinfI
730 740 750 760 770 780 790 800 810 820 830 840
TGTCTTGCTT CAAAAGATGT CATGCCCTTT GATTTGCTTA AAAAGAAGCT CTTGTTGAGA TTAGAGACAC TCGGAATCCG AATACTAAAG ACTTACGAAG AGGAATGGTC TTATATCCCA
>AluI
>BglII
>HinfI
>PstI
>Sau3AI
>AluI
850 860 870 880 890 900 910 920 930 940 950 960
GTAGGTGGTT CCTTGCCAAA CACGGAACAA AAGAATCTCG CCTTTGGTGC TGCAGCTAGC ATGGTTCATC CTGCAACAGG CTATTCAGTT GTGAGATCTT TGTCTGAAGC TCCAAAATAC
>AluI
>HindIII
>HaeIII
970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
GCATCAGTCA TCGCTAATAT ACTAAAACAT GAGACCACTA CTTCCTTCAC CAGACACATC AACACCAATA TTTCAAGACA AGCTTGGGAT ACTTTATGGC CACCAGAAAG GAAACGACAG
>AluI
>SpeI
>BamHI
>SacI
>HindIII
>EcoRI
>BstXI
>HaeIII
>Sau3AI
>AluI
>KpnI
>AluI
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
AGAGCATTCT TTCTAAGCCG AATTCCAGCA CACTGGCGGC CGTTACTAGT GGATCCGAGC TCGGTACCAA GCTTGGCGTA ATCATGGTCA TAGCTGTTTC CTGTGTGAAA TTGTTATCCG
>HspI
>AluI
1210 1220 1230 1240 1250 1260 1270
CTCACAATTC CACACAACAT ACGAGCCGGA AGCATAAAGT GTAAAGCCTG GGGTGCCTAA TGAGTGAGCT AA

FIGURE 10

```
                                                                    60
                                                                    *
GAGCTCGGAT CCACTAGTAA CGGCCGCCAG TGTGCTGGAA TTCGGCTTCT ATCTTGTACC

                                                                   120
                                                                    *
AAATTGTTGA TCATCTTAGC AAGAGGAACA GTTCCCTTCG TCATGATCTC CAACCTCGAG

                                                                   180
                                                                    *
GTATTAGAAG CATGCGAGAA GAGCGACAGC CCGAAGAACA CCAGGTCCGG GAGAAACAGC

                                                                   240
                                                                    *
CTCGACGACA AGAAACCATG CCAGTAACGC GGTTCCAGGT CAAAGAACGC ATCAAAGAAC

                                                                   300
                                                                    *
CTCCTAGTAG CATCCAAATC AAGCTTCAGC AAAATATCCA TCCCAAAACA GAAGAACTCC

                                                                   360
                                                                    *
CTCTGTCTCC GCCTCTCAAT AGGCCACAAG TCTCTCCACA CCTCAGCCGA GAGCTCATCT

                                                                   420
                                                                    *
CCTCTCAAGC CGTTGTTGTT ACCACCACCA AGGTACCGCA CTATAGCGTT TGCAACTATC

                                                                   480
                                                                    *
GGAGCAGCTG CAAGAGTCCT AGCAACCATG TAACCAGTCG AAGGATGAAC CATCCCCGCC
```

FIGURE 11

```
                                                                    540
                                                                     *
GTACCGCCAA TGCCAACAAC TCTTTGAGGC AAGACCGGTA AAGGACCTCC CATAGGGATC

                                                                    600
                                                                     *
ACACAACGCT CGTCTTCCTC AATCCGCTTC ACGTTGATCC CCAAATGTTT CAGCCTCGCA

                                                                    660
                                                                     *
ACCATCCTCT CTTGGATATC TTCCATCTTC AGACCCGGCC TAGCCACAAG AGACGTCTCT

                                                                    720
                                                                     *
TCAAGAAAGA TCCTGTTGGA AGAAAACGGC ATCGCGTACA GGAACGTAGG GATCTTGCTG

                                                                    780
                                                                     *
TTCCGCTCTT TAACCTCAGG GTACGCGTCA AGATGCTTAT CTCTCCAGTC CATGAACACC

                                                                   840
                                                                    *
ATCTTATCCA CATCAAACGG GTGACCATCG ACCTCAGCAA TGATACCATA AGCTACTTGA

                                                                    900
                                                                     *
TACCCAGGGT TATAAGGCTT ATCATACTGA ACCAAGCATC TTGAAAAACC AGTAGCGTCG

                                                                    960
                                                                     *
AGAACAACAG AAGCCTGAAT CTTCACACCG TCACTGCAGA CAACAGTGGA GTTAACCTCC
```

FIGURE 11

```
                                                                  1020
                                                                   *
TCGTGAACCA CGTCAGTGAC TTTAGCCTGA TGGAATCTAA CACCGTTGGT GATGCACTTC

                                                                  1080
                                                                   *
TGAAGCATCT TGGATTTGAG CTGTTTACGG TTCACTCTCC CGTAAGGCCG GGACAGGTCC

                                                                  1140
                                                                   *
TTTTCGGAGC CGTCGTTGAT GTAGACGACG GCGCCGGACC AGGTGGTGTC GAGGCAGTCT

                                                                  1200
                                                                   *
AGCAAGTCCA TGGCTTCGAA CTCGTCAACC CAAACTCCGT AGTTGTTAGG CCAAATGAGT

                                                                  1260
                                                                   *
TTGGGGGAAG GATCGATGGA GCAGACAGAG AGTCCAGCTT CGGAGACTTG CTGAGCCACG

                                                                  1320
                                                                   *
GCTAAACCAG CGGGGCCGCC GCCAACGATA GCTAGATCAA CAACTTTGTT CAGGGAAGTG

                                                                  1380
                                                                   *
TCGTTTAAAG GAAGGTCCAA GTCGAGATTC TCCTTCTTGG TTTCAGGAAC AAGATCCAAA

                                                                  1440
                                                                   *
AGAGCACTAC TAGCACTAGT GATACTACTA CCGATTCTGA TTGCTCTTTT CTTCAAACCA
```

FIGURE 11

```
                                                                    1500
                                                                     *
AGCTTAACCC TTGAAGGATT TGGACTTAAT CTCTCGAACC CATGAAACTG AGGGATGAAA

                                                                    1560
                                                                     *
AACTCGAGCT TGTTGGGTGT TTTCAACAGA GTATCCATCG AATTCTGCAG ATATCCATCA

CACTGGCGGC CGCTCGAGCA TGCATCTAGA
```

FIGURE 11

Sample ID #	Segregation ratio	Carotenoid concentration (µg/gFW) Lutein	Lycopene	α-Carotene	β-Carotene	Total
SP30021 control 1		24.4	ND	ND	1.9	26.3
SP30021 control 2		34.0	ND	ND	4.9	38.9
T2 3390-SP30021-1	3:1	33.5	6.1	229.0	385.7	654.3
T2 3390-SP30021-2	15:1	50.4	6.2	372.4	721.4	1150.4
T2 3390-SP30021-3	no fit	45.8	3.9	352.9	580.9	983.5
T2 3390-SP30021-4	3:1	31.0	4.9	306.1	463.3	805.3
T2 3390-SP30021-5	3:1	36.8	10.5	370.6	659.4	1077.3*
T2 3390-SP30021-6	15:1	46.9	9.1	445.1	797.0	1298.1
T2 3390-SP30021-7	15:1	51.2	7.4	494.9	941.4	1494.9
T2 3390-SP30021-8	no fit	41.9	11.3	468.4	904.3	1425.9
T2 3390-SP30021-9	>63:1	68.4	11.9	394.2	949.2	1423.7
T2 3390-SP30021-10	null	51.6	ND	12.6	22.8	87.0
T2 3390-SP30021-11	3:1	52.2	9.5	409.8	714.5	1186.0*
T2 3390-SP30021-12*	3:1	48.0	10.2	400.0	738.8	1197.0*
T2 3390-SP30021-13	3:1	66.1	3.9	98.1	216.0	384.1
T2 3390-SP30021-14	3:1	49.1	8.9	320.0	611.6	989.6
T2 3390-SP30021-15	null	27.0	ND	ND	1.2	28.2
T2 3390-SP30021-16	3:1	55.6	6.4	283.1	527.4	872.5
T2 3390-SP30021-17	3:1	53.0	9.1	324.9	614.3	1001.3
T2 3390-SP30021-18	>63:1	49.6	8.1	449.0	759.3	1266.0
T2 3390-SP30021-19	3:1	62.2	7.6	346.1	613.2	1029.1
T2 3390-SP30021-20	3:1	52.1	6.3	285.0	544.9	888.3
T2 3390-SP30021-21	3:1	56.2	4.1	187.9	334.2	582.4
T2 3390-SP30021-22	null	43.1	ND	ND	4.9	48.0
T2 3390-SP30021-23	3:1	71.0	10.9	358.6	693.9	1134.4*
T2 3390-SP30021-24	no fit	53.9	7.3	272.1	520.4	853.7
T2 3390-SP30021-25	3:1	31.9	12.2	309.1	580.9	934.1
T2 3390-SP30021-26*	3:1	34.3	9.3	311.2	584.4	939.2*
T2 3390-SP30021-27	3:1	52.6	9.8	299.8	686.3	1048.5*

FIGURE 12

T2 3390-SP30021-28	no fit	68.4	10.0	446.3	907.7	1432.4
T2 3390-SP30021-29	>63:1	85.1	8.5	459.4	822.9	1375.9
T2 3390-SP30021-30	3:1	63.7	5.8	356.9	598.4	1024.8
T2 3390-SP30021-31	3:1	76.0	7.3	302.5	527.1	912.9
T2 3390-SP30021-32	null	51.8	2.3	31.4	55.0	140.5
T2 3390-SP30021-33	3:1	36.3	8.9	283.1	546.9	875.2
T2 3390-SP30021-34	>63:1	86.9	12.1	502.3	808.3	1409.6
T2 3390-SP30021-35	3:1	39.3	8.1	224.5	461.0	732.9
T2 3390-SP30021-36	15:1	55.5	11.0	538.5	829.9	1434.9
T2 3390-SP30021-37*	3:1	50.3	10.0	291.1	625.9	977.3*
T2 3390-SP30021-38	3:1	70.5	8.1	309.0	576.1	963.7
T2 3390-SP30021-39	null	37.3	ND	ND	3.6	40.9
T2 3390-SP30021-40	3:1	37.5	1.8	251.1	505.2	796.0
T2 3390-SP30021-41	3:1	47.5	8.4	414.1	719.3	1189.3*
T2 3390-SP30021-42	3:1	42.6	5.1	230.3	352.9	630.9
T2 3390-SP30021-43	no fit	83.3	5.6	128.4	219.8	437.9
T2 3390-SP30021-46	3:1	21.6	1.4	211.2	368.3	602.5
T2 3390-SP30021-47	3:1	79.1	3.7	312.5	570.5	965.8
T2 3390-SP30021-48	3:1	45.3	3.0	225.2	401.5	675.0
T2 3390-SP30021-49	15:1	28.3	1.6	346.0	677.2	1053.1
T4 3390-SP001-1-6-13	Homo	52.4	1.5	439.5	669.3	1162.7

FIGURE 12

Sample ID #	Segregation status	Carotenoid concentration (μg/gFW)				
		Lutein	Lycopene	α-Carotene	β-Carotene	Total
T3 3390-SP001-4-12	Homo	43.9	17.2	282.1	636.8	980.0
T3 3390-SP001-5-7	Het	50.7	6.3	190.6	386.8	634.4
T3 3390-SP001-5-12	Homo	45.5	19.5	255.9	633.4	954.3
T3 3390-SP001-11-6	Homo	46.5	12.8	372.2	538.4	969.9
T3 3390-SP001-11-9	Homo	54.0	10.2	406.0	556.0	1026.2
T3 3390-SP001-14-2	Homo	59.7	12.5	342.4	764.0	1178.6
T3 3390-SP001-14-6	Homo	66.3	12.9	431.0	673.9	1184.1
T3 3390-SP001-15-9	Homo	30.8	14.3	271.8	559.8	876.7
T3 3390-SP001-15-12	Homo	39.6	13.1	241.7	649.1	943.5
T3 3390-SP001-16-3	Homo	49.9	17.1	230.2	519.7	816.9
T3 3390-SP001-16-6	Homo	35.5	21.1	263.8	547.7	868.1
T3 3390-SP001-35-2	Het	37.6	7.2	125.4	313.9	484.1
T3 3390-SP001-35-10	Homo	43.7	16.6	234.7	503.9	798.9
T3 3390-SP001-35-12	Homo	50.2	21.3	361.7	695.7	1128.9
T3 3390-SP001-8-3	Het	41.4	9.9	178.2	434.4	663.9
T3 3390-SP001-8-9	Homo	39.1	18.2	309.3	505.0	871.6
T3 3390-SP001-8-11	Homo	35.9	19.6	260.7	580.4	896.6
T3 3390-SP001-18-8	Het	29.2	12.2	112.1	247.6	441.1
T3 3390-SP001-16-10	Het	38.0	14.6	248.2	486.3	787.1
T4 3390-SP001-1-6-1	Homo	27.8	20.5	248.7	379.1	676.1
T4 3390-SP001-1-6-8	Homo	38.5	16.8	304.1	383.9	743.3
VAR SP001-4-5		54.2	ND	ND	5.8	60.0
VAR SP001-4-6		51.2	ND	ND	7.0	58.2
VAR SP001-4-10		30.2	ND	ND	ND	30.2

FIGURE 13

Sample ID #	Segregation ratio	Carotenoid concentration (µg/gFW) Lutein	Lycopene	α-Carotene	β-Carotene	Phytoene	Total
SP30021 control	Homo	21	ND	ND	2	ND	23
9002-SP30021-1*	3:1	20	2	394	618	210	1244
9002-SP30021-2	3:1	17	2	285	537	128	969
9002-SP30021-3	>64:1	19	7	489	689	381	1585
9002-SP30021-4	3:1	58	5	105	266	94	528
9002-SP30021-5	15:1	24	3	416	649	265	1357
9002-SP30021-6	3:1	13	2	324	546	176	1061
9002-SP30021-7	3:1	13	4	344	465	212	1038
9002-SP30021-8	15:1	12	3	449	690	224	1378
9002-SP30021-9	>64:1	24	5	499	724	313	1565
9002-SP30021-10	15:1	52	25	387	505	245	1214
9002-SP30021-11	3:1	29	2	301	480	187	999
9002-SP30021-12	>64:1	43	10	575	779	436	1843
9002-SP30021-13	3:1	19	3	357	509	279	1167
9002-SP30021-14	null	33	ND	ND	3	ND	36
9002-SP30021-15*	3:1	29	7	472	599	354	1461
9002-SP30021-16	64:1	40	3	315	436	203	997
9002-SP30021-17	15:1	25	7	322	467	144	967
9002-SP30021-18	>64:1	8	4	447	647	313	1419
9002-SP30021-19	15:1	38	17	537	570	327	1489
9002-SP30021-20*	3:1	32	8	363	629	173	1205
9002-SP30021-21	>64:1	1	6	468	736	348	1559
9002-SP30021-22	15:1	68	29	308	423	173	1001
9002-SP30021-23	15:1	51	20	449	553	423	1496
9002-SP30021-24	3:1	47	20	339	515	311	1232
9002-SP30021-25	null	27	ND	ND	2	ND	29
9002-SP30021-26*	3:1	4	3	346	605	150	1108
9002-SP30021-27	>64:1	25	5	416	698	376	1520
9002-SP30021-28	15:1	75	9	464	527	333	1408
9002-SP30021-29	null	32	ND	16	34	ND	82
9002-SP30021-30	3:1	25	9	316	525	182	1057
9002-SP30021-31	null	28	ND	ND	2	ND	30
9002-SP30021-32	3:1	29	5	198	283	132	647
9002-SP30021-33	15:1	50	40	408	557	324	1379
9002-SP30021-34	15:1	43	5	216	289	132	685
9002-SP30021-35	3:1	29	8	303	511	281	1132
9002-SP30021-36	3:1	26	9	324	402	157	918
9002-SP30021-37	3:1	34	11	263	418	143	869
9002-SP30021-39	15:1	54	13	219	420	118	824
9002-SP30021-40	15:1	30	7	382	716	235	1370
9002-SP30021-41	3:1	52	15	440	506	396	1409
9002-SP30021-42	3:1	49	20	317	516	170	1072
9002-SP30021-44	>64:1	34	7	368	647	310	1366
9002-SP30021-45	>64:1	45	9	429	636	402	1521
9002-SP30021-46	3:1	100	14	456	699	347	1617
9002-SP30021-48	3:1	37	5	191	354	231	818
9002-SP30021-50	64:1	51	22	522	756	303	1654

FIGURE 14

```
          10                  20                  30                  40
ATG GCT TCT ATG ATA TCC TCT TCC GCT GTG ACA ACA GTC AGC CGT
TAC CGA AGA TAC TAT AGG AGA AGG CGA CAC TGT TGT CAG TCG GCA
 M   A   S   M   I   S   S   S   A   V   T   T   V   S   R>

    50                  60                  70                  80                  90
GCC TCT AGG GGG CAA TCC GCC GCA GTG GCT CCA TTC GGC GGC CTC
CGG AGA TCC CCC GTT AGG CGG CGT CAC CGA GGT AAG CCG CCG GAG
 A   S   R   G   Q   S   A   A   V   A   P   F   G   G   L>

          100                 110                 120                 130
AAA TCC ATG ACT GGA TTC CCA GTG AAG AAG GTC AAC ACT GAC ATT
TTT AGG TAC TGA CCT AAG GGT CAC TTC TTC CAG TTG TGA CTG TAA
 K   S   M   T   G   F   P   V   K   K   V   N   T   D   I>

   140                 150                 160                 170                 180
ACT TCC ATT ACA AGC AAT GGT GGA AGA GTA AAG TGC ATG ACC AAC
TGA AGG TAA TGT TCG TTA CCA CCT TCT CAT TTC ACG TAC TGG TTG
 T   S   I   T   S   N   G   G   R   V   K   C   M   T   N>

          190                 200                 210                 220
TTC TTG ATC GTT GTC GCT ACC GTT TTG GTT ATG GAG TTG ACT GCT
AAG AAC TAG CAA CAG CGA TGG CAA AAC CAA TAC CTC AAC TGA CGA
 F   L   I   V   V   A   T   V   L   V   M   E   L   T   A>

   230                 240                 250                 260                 270
TAC TCC GTC CAC AGA TGG ATC ATG CAC GGT CCA TTG GGT TGG GGT
ATG AGG CAG GTG TCT ACC TAG TAC GTG CCA GGT AAC CCA ACC CCA
 Y   S   V   H   R   W   I   M   H   G   P   L   G   W   G>
```

FIGURE 15

```
          280         290         300         310
TGG CAC AAG TCC CAC CAC GAG GAG CAC GAC CAC GCT TTG GAG AAG
ACC GTG TTC AGG GTG GTG CTC CTC GTG CTG GTG CGA AAC CTC TTC
 W   H   K   S   H   H   E   E   H   D   H   A   L   E   K>

   320         330         340         350         360
AAC GAC TTG TAC GGT TTG GTT TTC GCT GTT ATC GCT ACC GTC TTG
TTG CTG AAC ATG CCA AAC CAA AAG CGA CAA TAG CGA TGG CAG AAC
 N   D   L   Y   G   L   V   F   A   V   I   A   T   V   L>

          370         380         390         400
TTC ACC GTT GGT TGG ATC TGG GCT CCA GTT TTG TGG TGG ATC GCT
AAG TGG CAA CCA ACC TAG ACC CGA GGT CAA AAC ACC ACC TAG CGA
 F   T   V   G   W   I   W   A   P   V   L   W   W   I   A>

   410         420         430         440         450
TTG GGT ATG ACT GTC TAC GGT TTG ATC TAC TTC GTT TTG CAC GAT
AAC CCA TAC TGA CAG ATG CCA AAC TAG ATG AAG CAA AAC GTG CTA
 L   G   M   T   V   Y   G   L   I   Y   F   V   L   H   D>

          460         470         480         490
GGT TTG GTC CAC CAA AGA TGG CCA TTC AGA TAC ATC CCA AGA AAG
CCA AAC CAG GTG GTT TCT ACC GGT AAG TCT ATG TAG GGT TCT TTC
 G   L   V   H   Q   R   W   P   F   R   Y   I   P   R   K>

   500         510         520         530         540
GGT TAC GCT AGA AGA TTG TAC CAA GCT CAC AGA TTG CAC CAC GCT
CCA ATG CGA TCT TCT AAC ATG GTT CGA GTG TCT AAC GTG GTG CGA
 G   Y   A   R   R   L   Y   Q   A   H   R   L   H   H   A>
```

FIGURE 15

```
          550         560         570         580
GTC GAG GGT AGA GAC CAC TGT GTT TCT TTC GGT TTC ATC TAC GCT
CAG CTC CCA TCT CTG GTG ACA CAA AGA AAG CCA AAG TAG ATG CGA
 V   E   G   R   D   H   C   V   S   F   G   F   I   Y   A>

  590         600         610         620         630
CCA CCA GTT GAT AAG TTG AAG CAA GAC TTG AAG ATG TCC GGT GTC
GGT GGT CAA CTA TTC AAC TTC GTT CTG AAC TTC TAC AGG CCA CAG
 P   P   V   D   K   L   K   Q   D   L   K   M   S   G   V>

          640         650
TTG AGA GCT GAG GCT CAA GAG AGA ACC TAG
AAC TCT CGA CTC CGA GTT CTC TCT TGG
 L   R   A   E   A   Q   E   R   T>
```

FIGURE 15

```
                10                  20                  30                  40
ATG GCT TCT ATG ATA TCC TCT TCC GCT GTG ACA ACA GTC AGC CGT
TAC CGA AGA TAC TAT AGG AGA AGG CGA CAC TGT TGT CAG TCG GCA
 M   A   S   M   I   S   S   S   A   V   T   T   V   S   R>

    50                  60                  70                  80                  90
GCC TCT AGG GGG CAA TCC GCC GCA GTG GCT CCA TTC GGC GGC CTC
CGG AGA TCC CCC GTT AGG CGG CGT CAC CGA GGT AAG CCG CCG GAG
 A   S   R   G   Q   S   A   A   V   A   P   F   G   G   L>

            100                 110                 120                 130
AAA TCC ATG ACT GGA TTC CCA GTG AAG AAG GTC AAC ACT GAC ATT
TTT AGG TAC TGA CCT AAG GGT CAC TTC TTC CAG TTG TGA CTG TAA
 K   S   M   T   G   F   P   V   K   K   V   N   T   D   I>

   140                 150                 160                 170                 180
ACT TCC ATT ACA AGC AAT GGT GGA AGA GTA AAG TGC ATG TCC GCT
TGA AGG TAA TGT TCG TTA CCA CCT TCT CAT TTC ACG TAC AGG CGA
 T   S   I   T   S   N   G   G   R   V   K   C   M   S   A>

            190                 200                 210                 220
CAC GCT TTG CCA AAG GCT GAC TTG ACT GCT ACC TCC TTG ATC GTC
GTG CGA AAC GGT TTC CGA CTG AAC TGA CGA TGG AGG AAC TAG CAG
 H   A   L   P   K   A   D   L   T   A   T   S   L   I   V>

   230                 240                 250                 260                 270
TCC GGT GGT ATC ATC GCT GCT TGG TTG GCT TTG CAC GTT CAC GCT
AGG CCA CCA TAG TAG CGA CGA ACC AAC CGA AAC GTG CAA GTG CGA
 S   G   G   I   I   A   A   W   L   A   L   H   V   H   A>

            280                 290                 300                 310
TTG TGG TTC TTG GAC GCT GCT GCT CAC CCA ATC TTG GCT ATC GCT
AAC ACC AAG AAC CTG CGA CGA CGA GTG GGT TAG AAC CGA TAG CGA
 L   W   F   L   D   A   A   A   H   P   I   L   A   I   A>
```

FIGURE 16

```
   320           330           340           350           360
AAC TTC TTG GGT TTG AAC TGG TTG TCT GTC GGT TTG TTC ATC ATC
TTG AAG AAC CCA AAC TTG ACC AAC AGA CAG CCA AAC AAG TAG TAG
 N   F   L   G   L   N   W   L   S   V   G   L   F   I   I>

         370           380           390           400
GCT CAC GAC GCT ATG CAC GGT TCC GTT GTC CCA GGT AGA CCA AGA
CGA GTG CTG CGA TAC GTG CCA AGG CAA CAG GGT CCA TCT GGT TCT
 A   H   D   A   M   H   G   S   V   V   P   G   R   P   R>

   410           420           430           440           450
GCT AAC GCT GCT ATG GGT CAA TTG GTT TTG TGG TTG TAC GCT GGT
CGA TTG CGA CGA TAC CCA GTT AAC CAA AAC ACC AAC ATG CGA CCA
 A   N   A   A   M   G   Q   L   V   L   W   L   Y   A   G>

         460           470           480           490
TTC TCT TGG AGA AAG ATG ATC GTT AAG CAC ATG GCT CAC CAC AGA
AAG AGA ACC TCT TTC TAC TAG CAA TTC GTG TAC CGA GTG GTG TCT
 F   S   W   R   K   M   I   V   K   H   M   A   H   H   R>

   500           510           520           530           540
CAC GCT GGT ACT GAT GAC GAC CCA GAT TTC GAC CAC GGT GGT CCA
GTG CGA CCA TGA CTA CTG CTG GGT CTA AAG CTG GTG CCA CCA GGT
 H   A   G   T   D   D   D   P   D   F   D   H   G   G   P>

         550           560           570           580
GTT AGA TGG TAC GCT AGA TTC ATC GGT ACT TAC TTC GGT TGG AGA
CAA TCT ACC ATG CGA TCT AAG TAG CCA TGA ATG AAG CCA ACC TCT
 V   R   W   Y   A   R   F   I   G   T   Y   F   G   W   R>

   590           600           610           620           630
GAG GGT TTG TTG TTG CCA GTC ATC GTT ACC GTT TAC GCT TTG ATC
CTC CCA AAC AAC AAC GGT CAG TAG CAA TGG CAA ATG CGA AAC TAG
 E   G   L   L   L   P   V   I   V   T   V   Y   A   L   I>
```

FIGURE 16

```
          640        650        660        670
TTG GGT GAC AGA TGG ATG TAC GTT GTC TTC TGG CCA TTG CCA TCC
AAC CCA CTG TCT ACC TAC ATG CAA CAG AAG ACC GGT AAC GGT AGG
 L   G   D   R   W   M   Y   V   V   F   W   P   L   P   S>

   680        690        700        710        720
ATC TTG GCT TCT ATC CAA TTG TTC GTT TTC GGT ACC TGG TTG CCA
TAG AAC CGA AGA TAG GTT AAC AAG CAA AAG CCA TGG ACC AAC GGT
 I   L   A   S   I   Q   L   F   V   F   G   T   W   L   P>

          730        740        750        760
CAC AGA CCA GGT CAC GAC GCT TTC CCA GAC AGA CAC AAC GCT CGA
GTG TCT GGT CCA GTG CTG CGA AAG GGT CTG TCT GTG TTG CGA GCT
 H   R   P   G   H   D   A   F   P   D   R   H   N   A   R>

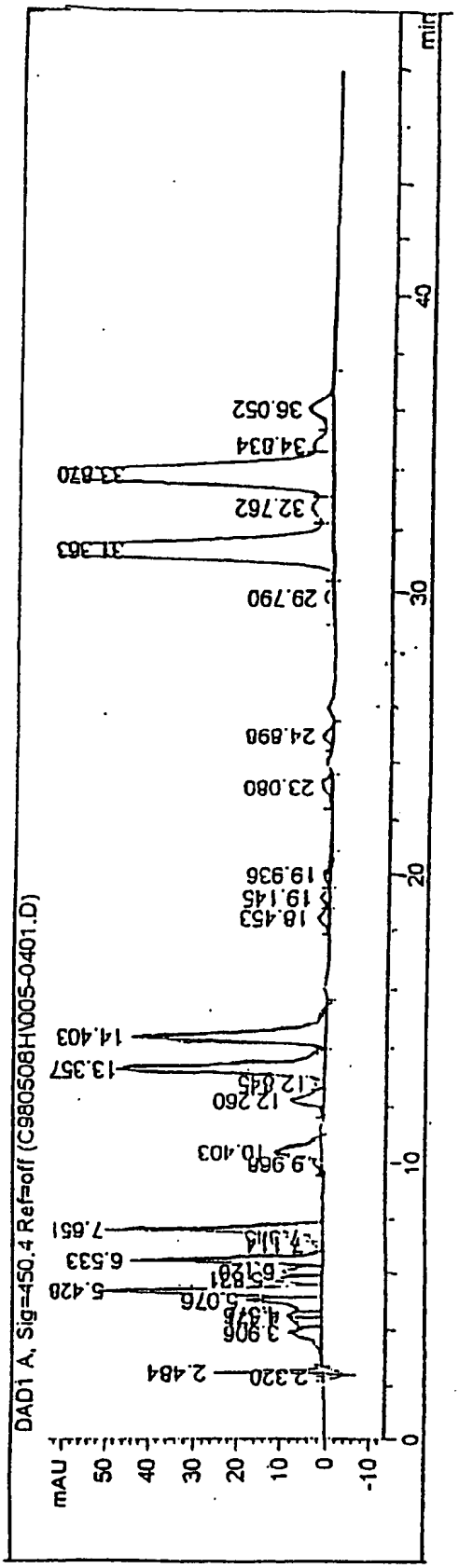
   770        780        790        800        810
TCC TCC AGA ATC TCT GAT CCA GTT TCC TTG TTG ACC TGT TTC CAC
AGG AGG TCT TAG AGA CTA GGT CAA AGG AAC AAC TGG ACA AAG GTG
 S   S   R   I   S   D   P   V   S   L   L   T   C   F   H>

          820        830        840        850
TTC GGT GGT TAC CAC CAC GAG CAC CAC TTG CAC CCA ACT GTC CCA
AAG CCA CCA ATG GTG GTG CTC GTG GTG AAC GTG GGT TGA CAG GGT
 F   G   G   Y   H   H   E   H   H   L   H   P   T   V   P>

   860        870        880        890        900
TGG TGG AGA TTG CCA TCC ACC AGA ACC AAG GGT GAC ACC GCT TAG
ACC ACC TCT AAC GGT AGG TGG TCT TGG TTC CCA CTG TGG CGA ATC
 W   W   R   L   P   S   T   R   T   K   G   D   T   A   *>

TA
AT
```

FIGURE 16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20
30
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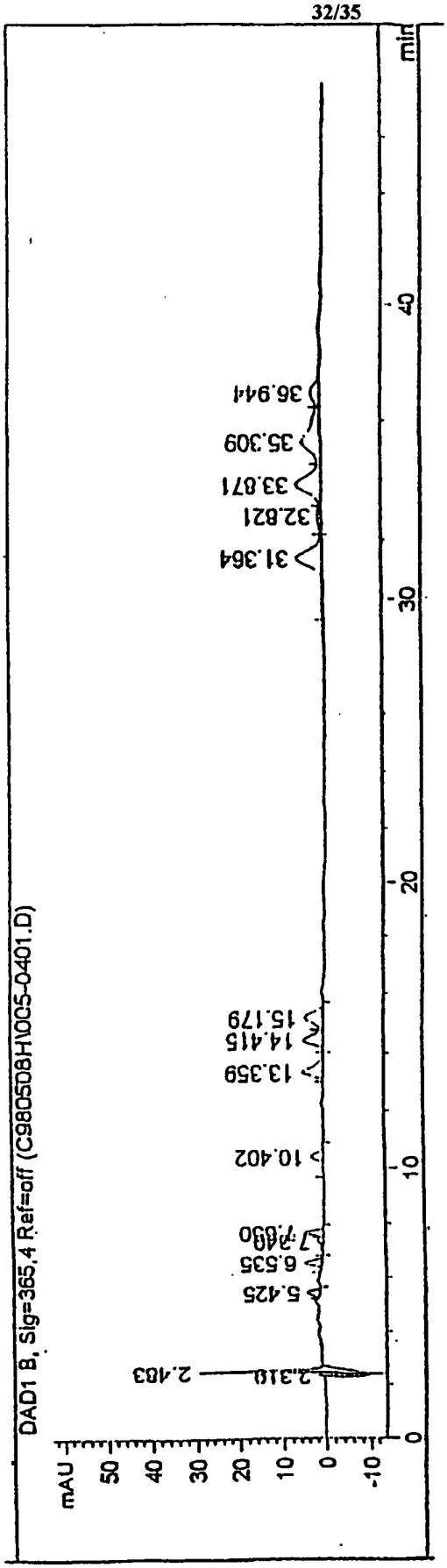
2.484
3.906
4.375
5.076
5.420
5.821
6.120
6.533
7.113
7.651
9.968
10.403
12.260
12.845
13.357
14.403
18.453
19.145
19.936
23.080
24.890
29.790
31.363
32.762
33.870
34.834
36.052

FIGURE 17A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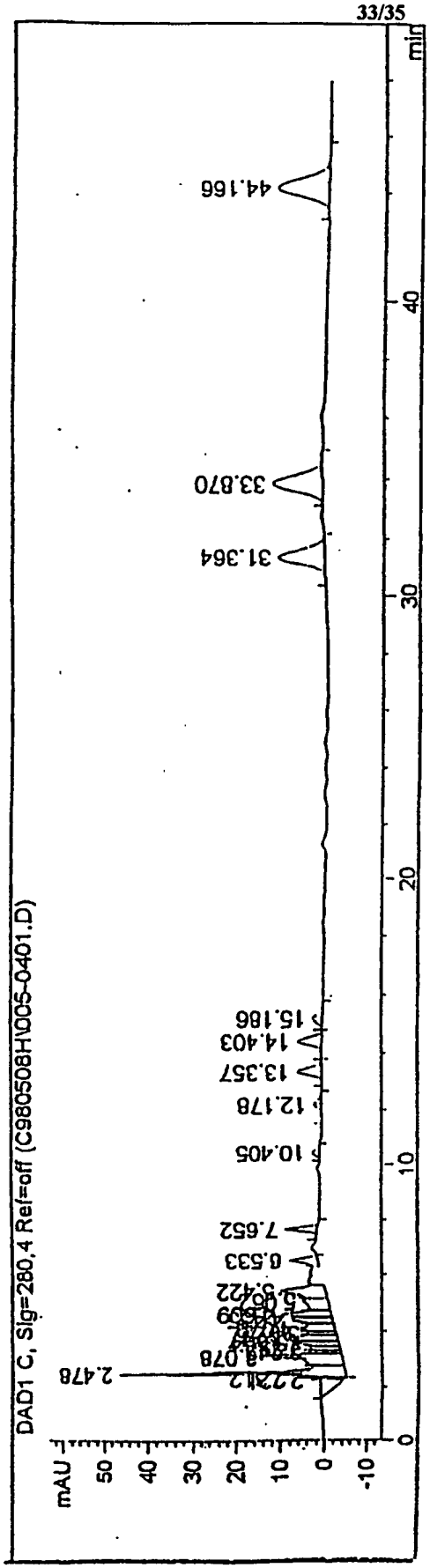
-10
min
0
10
20
30
40
2.310
2.403
5.425
6.535
7.340
7.850
10.402
13.359
14.415
15.179
31.364
32.821
33.871
35.309
36.941

FIGURE 17B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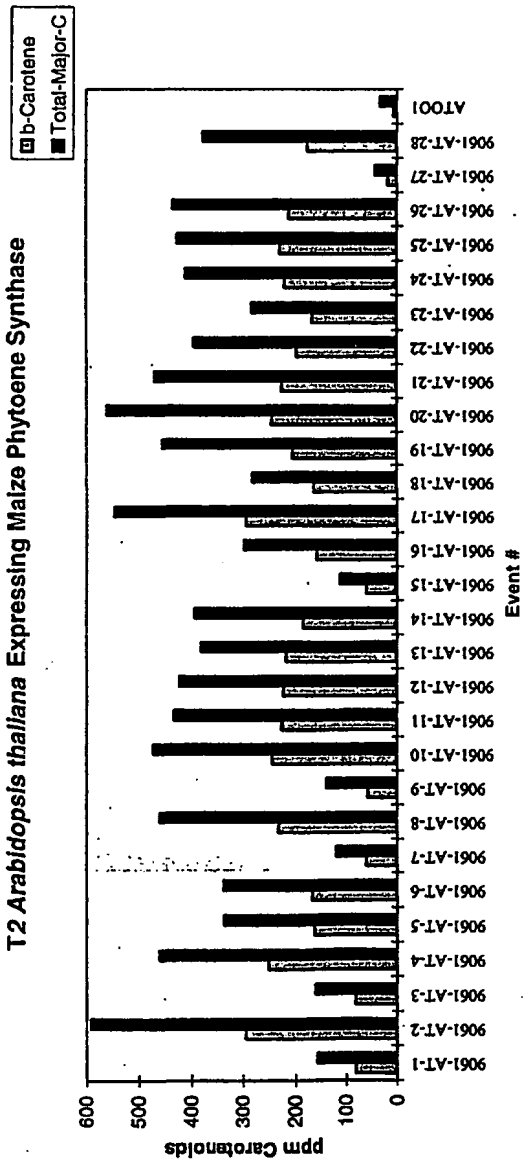
15.186
14.403
13.357
12.178
10.405
7.652
6.533

FIGURE 17C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9061-AT-7
9061-AT-6
9061-AT-5
9061-AT-4
9061-AT-3
9061-AT-2
9061-AT-1

Figure 18

```
   1  AATTCGCCCT TCCTCCTCGA GCGGGATCCA TGGCCATCAT ACTCGTACGA
  51  GCAGCGTCGC CGGGGCTCTC CGCCGCCGAC AGCATCAGCC ACCAGGGGAC
 101  TCTCCAGTGC TCCACCCTGC TCAAGACGAA GAGGCCGGCG GCGCGCCGGT
 151  GGATGCCCTG CTCGCTCCTT GGCCTCCACC CGTGGGAGGC TGGCCGTCCC
 201  TCCCCCGCCG TCTACTCCAG CCTCGCCGTC AACCCGGCGG GAGAGGCCGT
 251  CGTCTCGTCC GAGCAGAAGG TCTACGACGT CGTGCTCAAG CAGGCCGCAT
 301  TGCTCAAACG CCAGCTGCGC ACGCCGGTCC TCGACGCCAG GCCCCAGGAC
 351  ATGGACATGC CACGCAACGG GCTCAAGGAA GCCTACGACC GCTGCGGCGA
 401  GATCTGTGAG GAGTATGCCA AGACGTTTTA CCTCGGAACT ATGTTGATGA
 451  CAGAGGAGCG GCGCCGCGCC ATATGGGCCA TCTATGTGTG GTGTAGGAGG
 501  ACAGATGAGC TTGTAGATGG GCCAAACGCC AACTACATTA CACCAACAGC
 551  TTTGGACCGG TGGGAGAAGA GACTTGAGGA TCTGTTCACG GGACGTCCTT
 601  ACGACATGCT TGATGCCGCT CTCTCTGATA CCATCTCAAG GTTCCCCATA
 651  GACATTCAGC CATTCAGGGA CATGATTGAA GGGATGAGGA GTGATCTTAG
 701  GAAGACAAGG TATAACAACT TCGACGAGCT CTACATGTAC TGCTACTATG
 751  TTGCTGGAAC TGTCGGGTTA ATGAGCGTAC CTGTGATGGG CATCGCAACC
 801  GAGTCTAAAG CAACAACTGA AAGCGTATAC AGTGCTGCCT TGGCTCTGGG
 851  AATTGCGAAC CAACTCACGA ACATACTCCG GGATGTTGGA GAGGATGCTA
 901  GAAGAGGAAG GATATATTTA CCACAAGATG AGCTTGCACA GGCAGGGCTC
 951  TCTGATGAGG ACATCTTCAA AGGGGTCGTC ACGAACCGGT GGAGAAACTT
1001  CATGAAGAGG CAGATCAAGA GGGCCAGGAT GTTTTTTGAG GAGGCAGAGA
1051  GAGGGGTAAA TGAGCTCTCA CAGGCTAGCA GATGGCCAGT ATGGGCTTCC
1101  CTGTTGTTGT ACAGGCAGAT CCTGGATGAG ATCGAAGCCA ACGACTACAA
1151  CAACTTCACG AAGAGGGCGT ATGTTGGTAA AGGGAAGAAG TTGCTAGCAC
1201  TTCCTGTGGC ATATGGAAAA TCGCTACTGC TCCCATGTTC ATTGAGAAAT
1251  GGCCAGACCT AGCCACCAGA GAAGCTGCAG GATCCTCCTC GAGACTGAAG
1301  GGCG
```

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WRNFMKRQIKRARMFFEEAERGVNELSQASRWPVWASLLLYRQILDEIEANDYNNFTKRAYVGKGKKLLALPVAYGKSLL
LPCSLRNGQT*RPEKLQDPPRD*RA
```

Figure 19